# BEHAVIOURAL REACTIONS OF RATS TO DIFFERENT DRUGS FOLLOWING PERINATAL EXPOSURE TO CAFFEINE

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# ABSTRACT

Rats previously exposed to caffeine during both gestation and lactation at a low and high dose and saline-treated animals were given five drugs intraperitoneally in successive weeks. Rats were divided into two equal groups, the first receiving saline, diazepam (a benzodiazepine) at low and high dose and caffeine at low and high dose. The second group were given saline, Chlorohexyladenosine (CHA, an adenosine agonist) and oxprenolol (a beta-blocker). After drug administration each rat was tested in an open field and emergence apparatus.

Greater activity was found with caffeine doses, but lower activity and more grooming in the high perinatal caffeine group. Sedation was the main effect with diazepam at high doses and CHA at both doses, while oxprenolol had a slight stimulatory effect on activity. Increased centre occupancy was found with all three of these drugs in the high perinatal caffeine group but only in the CHA group did this interact with the high dose given.

None of the drugs had a marked effect on behaviour of rats but the implication is that anti-anxiety effects may have occurred for all three drugs but was more marked with CHA. This supports a role for adenosine in caffeine's effects on behaviour.

This is in agreement with research suggesting that adenosine is responsible for caffeine's anxiogenic role, and research showing an upregulation of adenosine receptors after chronic caffeine exposure. This finding is tentative, more work is required to discover the mechanisms responsible for caffeine's effects.

# CHAPTER ONE
# INTRODUCTION

The common use and general acceptance of coffee in today's society has led to a situation where caffeine has become a potential drug of abuse. Its stimulant properties have led to an overuse as people rely on it to keep them awake and alert. A high percentage of the population drink tea or coffee and those who do not may be exposed to caffeine in soft drinks or chocolate and other cocoa products, its use in many products such as medicines has made it possible for people to be unaware of the amount of caffeine they are consuming. Because it is readily available and consumed by a large number of people worldwide, it is considered harmless and therefore most people do not concern themselves with the possible negative effects from drinking it in large quantities or while pregnant and/or breast-feeding.

However, increasing concern about this particular issue has developed and is the concern of this thesis. I would like to give a brief description of caffeine and the effect it has as a stimulant as well as the effects with higher chronic consumption. Caffeine has a number of effects on the body which, at low doses may not be particularly harmful but with a high dose, can be fully expressed in a detrimental way. This can lead to problems which may result in one being diagnosed with psychiatric disorders and prescribed drugs to combat a condition which is reversible simply by reducing the amount of caffeine that is being consumed.

These physiological and psychological effects of caffeine will be explored. Of great interest is caffeine's teratogenic potential, both behaviourally and physically, which may be long-lasting. This will be examined in humans, for whom research is limited due to the ethics and retrospective problems which exist, and in animal research which will help

to understand the effects that caffeine has when it acts on the foetus and lactating animals. An examination of the theories for caffeine's actions will then be undertaken with a presentation of the research carried out in this area. Some hypotheses that emerge from the research will be proposed leading to the rationale for my study.

## CAFFEINE'S PHYSIOLOGICAL EFFECTS

Caffeine is a xanthine, known as 1,3,7 trimethylxanthine. It is a central nervous system (CNS) stimulant along with theobromine which is also in the xanthine family. Caffeine is contained in a number of items such as the obvious coffee and tea but also in Coca cola, chocolate bars, cocoa and in medications such as Nodoz and Anacin. The amount of caffeine in each of the beverages varies greatly (Table one). A 5 oz cup of brewed coffee can contain between 40 and 180 milligrams (mg) of caffeine while a cup of instant coffee can contain between 30 and 120 mg (Sobotka,1989).

This wide variability can make the calculation of caffeine intake difficult to assess, particularly when relying on a person's self report, therefore studies assessing effects on subjects after specified doses are provided are more reliable.

Stimulant effects occur at doses of around 2 - 4 mg/kg which is about one or two cups of coffee for most people. At doses higher than 5 mg/kg adverse effects to caffeine develop and withdrawal is usually experienced when chronic consumption of this amount occurs (Weiss and Laties, 1962). When caffeine ingestion reaches greater than 1000 mg, caffeinism occurs. However, lethal doses are very high, over 15 grams which is far higher than

would normally be ingested.

Table 1: Caffeine content of common commodities

| Product | Average mg. caffeine | Range |
|---|---|---|
| Coffee - brewed | 98 | 40 - 180 |
| -instant | 65 | 30 - 120 |
| Tea - brewed | 50 | 20 - 110 |
| - instant/iced | 30 | 25 - 76 |
| Cocoa beverage (5 oz) | 4 | 2 - 26 |
| Chocolate (1 oz) | 14 | 135 |
| Soft drink (12 oz) | 40 | 1 - 59 |
| Prescription drugs | 51 | 32 - 100 |
| Weight control aids | 170 | 100 - 200 |
| Alertness tablets | 150 | 100 - 200 |
| Analgesic/pain relief | 41 | 32 - 65 |
| Diuretics | 167 | 100 - 200 |
| Cold/ allergy remedies | 27 | 16 - 30 |

Taken from Sobotka (1989) adapted from Lecos (1984)

There are many physiological effects of caffeine. It is known that the xanthines affect firstly the cortex and then the medulla, with large doses (500 mg or more) stimulating the spinal cord. Many different systems and organs in the body are affected by caffeine consumption. These systems are the nervous system, urinary system, gastrointestinal system, cardiovascular system, respiratory system, endocrine and metabolic processes as well as the skin and appendages, so the effects are certainly widespread.

There is a great deal of literature describing the effects of caffeine on the cardiovascular system. Increases in blood pressure have been reported

with acute ingestion of 150mg of caffeine (Ellinwood and Rockwell, 1988) but this effect is still inconclusive. Although there have been a few supportive cases for this (Robertson et al. 1978), a specific relationship between caffeine consumption and blood pressure has not been found.

Caffeine stimulates the cardiac muscle causing an increase in the force of contraction, heart rate and cardiac output. In the other direction the medullary vagal muscle is also stimulated which has the opposite effect by decreasing the heart rate so that it can negate the increase by the cardiac muscle. As a result, bradycardia, tachycardia or no change may occur. Caffeine has also been implicated in myocardial infarction but there is little evidence for this. Cardiac arrhythmias also seem to be more common with high caffeine consumers (Greden, 1974) but the association has not been extensively studied.

Coronary heart disease was found to be twice or three times more likely in men ingesting five or more cups of coffee per day according to La Croix et al. (1986). However other researchers ( James and Stirling, 1983) did not find this relationship.

Gastrointestinal effects are another problem with caffeine ingestion. Nausea and vomiting can occur with large doses and hydrochloric acid secretion is stimulated by caffeine (Eilenhorn and Barceloux, 1988). As a result, stomach ulcers are more likely to occur. Decaffeinated coffee also stimulates gastric secretion, little is known about the effects of substances in coffee other than caffeine, therefore there may be another chemical in coffee causing deleterious effects (Curatalo and Robertson, 1983).

Carcinogenic effects have also been proposed to occur with caffeine consumption athough this has not been well established. Pancreatic cancer has been the most suspected outcome but kidney and lower urinary tract cancer have also been suggested with seven or more cups of coffee per day

(James and Stirling, 1983). However there is no conclusive evidence for the relationship.

The urinary system is affected by caffeine with both increased volume of urine and sodium excretion. This occurs because of a decrease in tubular resorption of sodium and water which can lead to a loss of fluids and electrolytes (Eilenhorn and Barceloux, 1988). Tolerance apparently develops to this response with chronic consumption.

Metabolism is also affected by caffeine intake, Eilenhorn and Barceloux report that increases occur in metabolism with blood glucose levels (hyperglycemia) increasing as well. However, this appears to occur only in caffeine-naive people since tolerance develops in the chronic consumer. Curatolo and Robertson (1983) reported that an increase in free fatty acids 50 to 100 per cent occurs with acute caffeine. Respiratory rates are also known to be affected by caffeine. The medullary respiratory centre is stimulated which causes an increase in respiratory rate, oxygen consumption and carbon dioxide elimination (Sawyer, Julia and Turin, 1982).

Another effect of caffeine can be to change sleep patterns. Sleep onset is delayed (Revelle et al., 1980) and the quality of sleep is reduced at amounts of around 100 mg or greater by increasing stage two sleep, decreasing stage three and four and having variable effects on REM sleep (Curatolo and Robertson,1983). Again, tolerance develops to caffeine and sleep is less disrupted when chronically consumed.

Effects on the nervous system are the most interesting for this study, and can be quite extreme ranging from problems of tremor, headache, anxiety, tenseness and irritability to caffeine psychosis with schizophrenic-type symptoms. A condition termed caffeinism can also occur. Doses required for this need to be fairly high. Most reported cases have been

higher than 1000 mg. A greater description of caffeinism will be given later when discussing the psychological effects of caffeine.

## FACTORS MEDIATING CAFFEINE'S EFFECTS

Some important factors which mediate the effects of caffeine must also be mentioned to understand the full effects that caffeine has on the body. These fall into the categories of tolerance, dependence, withdrawal and sensitivity to caffeine.

Tolerance to caffeine appears to be an important factor in caffeine's effects. Examples of metabolic, urinary and sleep problems disappearing with chronic caffeine use exemplifies this. The mechanism for this caffeine tolerance however is not well understood. It has been suggested that an upregulation of adenosine receptors is the reason for tolerance developing. Boulenger et al. (1983) found that there was an increase in the number of adenosine receptors with tolerance. However, Holtzman, Mante and Minneman (1991) found that with tolerance developing to caffeine, there was no evidence for it being related to an increase in adenosine receptors. The adenosine antagonist activity of caffeine did not decrease and there was no difference between control and caffeine-treated rats in regard to adenosine binding sites in cerebral cortex despite increases in receptors. More work needs to be done to discover how the tolerance mechanism does work.

The second factor influencing caffeine's effects concerns dependence.This is related to withdrawal as well. Both are characteristic of the heavy caffeine consumer. A physiological need often develops if the individual does not have their regular dose of caffeine. They will experience withdrawal, just as other drug abusers do when giving up an addiction. Withdrawal for the caffeine addict involves an onset at 12 to 24

hours after the last caffeine intake, peaks at 20 to 48 hours, and lasts about a week on average (Griffiths et al., 1988). Withdrawal involves a painful, generalized headache with associated discomfort including irritability, nervousness, decreased alertness, fatigue and sleepiness (Bruce et al., 1991). With caffeine consumption the symptoms disappear which clearly shows why people continue to ingest high quantities rather than abstain.

Coffee drinking is a habit, like smoking, that many people are not willing to change. It is difficult for them to do so since the morning cup of coffee is an integral part of their day, and coffee and tea drinking are also socially learned habits. Heavy consumers internally regulate the amount of caffeine they are drinking. Without consciously knowing they are doing so, they will increase the number of drinks they have if the caffeine content is lowered (Kozlowski, 1976).

Sensitivity to caffeine also influences caffeine use. Individual differences exist in the responses to caffeine. One person may be able to drink six cups of coffee before any noticeable effects occur whereas another may only need three or four. This makes quantifying the effects of certain amounts of caffeine difficult, although taking body weights into account does improve this and is a far better indicator than simply referring to the effects of four cups of coffee. Sensitivity to caffeine must be taken into account when considering the tolerance effect which develops. It is easy to attribute a lack of caffeine effects to tolerance when in fact it is just individual differences that are responsible (Goldstein, Warren and Kaiser, 1965). Perhaps heavy caffeine drinkers are not as sensitive to caffeine and can drink a lot more without any effects whereas those who do not drink very much caffeine do not because it does affect them more. This point has not been well studied, but tolerance is usually regarded as the reason for a lack of effects.

# PSYCHOLOGICAL EFFECTS OF CAFFEINE

The psychological effects of caffeine have been well documented and fall into the categories of performance, arousal, memory and mood.

## EFFECTS ON PERFORMANCE

Caffeine improves mental and physical performance (Weiss and Laties, 1962). A review of a wide range of activities showed that caffeine can enhance many behaviours, such as working to exhaustion on a bicycle ergometer, all day hikes, driving a truck for 18 to 20 hours per day, athletic track events and motor coordination tasks. Sleepiness and motor tests were both enhanced with caffeine treatment compared with placebo but the effect was greater for sleepiness. Lokes, Hinrichs and Ghoneim (1985) reported that caffeine impaired fine motor coordination tasks because of hand steadiness. Weiss and Laties related the lack of hand steadiness to psychomotor agitation causing unsteady hand movements. Curatolo and Robertson (1983) also found that at low and high doses, caffeine may impair performance on complex motor tasks and fine motor coordination tasks. Reaction time appears to be reduced after 24 hours which may be due to CNS depression after the caffeine effects wear off (Cheney, 1936, Horst and Jenkins, 1935).

Simple tasks such as arithmetic problems,typing and car driving which are all simple tasks, seem to be improved by caffeine at low doses (Regina et al., 1974). Other positive effects of caffeine include endurance and work capacity improvement but when the caffeine wears off, the countereffect of nervousness, irritability, sleep disturbance and fatigue

follows (Weiss and Laties, 1962).

It appears that the effects on performance by caffeine are interactive and complex, performance has been found to be influenced by personality (Gilliland, 1980; Revelle et al. (1980). Different personality types may respond differently to caffeine.

Increased alertness and wakefulness have been found with caffeine due to its stimulant effects. This may be dependent however on whether the person is a chronic caffeine consumer or not. Those that are caffeine naive experience more unpleasant effects and nervousness (Goldstein, Kaiser and Whitby, 1969). Sleep disturbances may also occur as described earlier.

Effects of caffeine on memory are not well understood. Terry and Phifer (1986) have suggested that longterm memory may be altered by caffeine while Erikson (1985) thought that encoding the manipulation of information may be impaired.

## EFFECTS ON MOOD

The effects on mood fall into the areas of anxiety and depression. Most studies on caffeine have been on acute effects and not many have considered chronic caffeine consumption which may be the more harmful. Anxiety can occur with either acute or chronic caffeine at high doses. Depression is usually associated with any anxiety effects. Panic anxiety in particular has been suggested to be affected by caffeine consumption as panic attacks have been found with high caffeine consumption (Charney, Heninger and Jatlow, 1985; Uhde et al., 1984).

# CAFFEINISM

Many studies have reported a relationship between high caffeine consumption and anxiety (Greden, 1974; MacCallum, 1979; Rapoport et al., 1984). Doses of 500 mg per day (approximately 4 to 7 cups of coffee) or more can lead to symptoms of caffeinism which can be indistinguishable from anxiety neurosis.

Greden (1974) reports three case studies of caffeinism, following consumption of 1000 mg or more per day. All three had symptoms of anxiety, tremulousness and headache. One had an irregular heartbeat while another suffered from insomnia. When they lowered their caffeine intake, scale ratings on the Hamilton Anxiety Scale returned to normal and the other symptoms also disappeared. All three went through withdrawal and when challenged after abstinence with high doses of caffeine, their anxiety returned. MacCallum (1979) published the case of a woman consuming 20 or more cups of coffee per day (well over one gram) who complained of anxiety, panic attacks, cold sweat attacks, shortness of breath and a floating sensation. With the cessation of coffee drinking, the anxiety disappeared and she was left with the withdrawal headache and some dizziness.

In addition to case studies, some empirical studies have been published. For example, Greden et al. (1978) looked at anxiety and depression in psychiatric patients. The State-Trait-Anxiety Index and the Beck Depression Scale were given to patients ranked as low consumers (0-249 mg caffeine per day), moderate consumers (250-749 mg per day and high consumers (750 mg or more per day). The high consumers scored higher on the two measures and they also reported feeling more symptoms than the other consumers, and that they did not think their health was as good. They also reported using more sedative-hypnotics and minor tranquillisers.

A study by Gilliland and Andress (1981) looked for symptoms of caffeinism in psychology students. They put the students into categories of abstainers, low, moderate and high consumers. They administered questionnaires on the effects of caffeine, psychophysiological symptoms, state-trait anxiety, and depression. Moderate and high consumers reported more deleterious effects and the high consumers reported more psychophysiological reactions. Higher trait anxiety scores were obtained for the moderate and moderate and high combined and Semester grade point averages were higher in the abstainers than in the high consumer group. Shanahan and Hughes (1986) found that performance anxiety was higher in chronic caffeine drinkers given acute caffeine than it was in coffee drinkers given decaffeinated drinks. Veleber and Templer (1984) also found that subjects given a high dose of caffeine, 300 mg per 45.36 kg body weight scored higher on an anxiety and depression scale than those with a lower dose (150 mg per kg body weight) or no caffeine.

Smith (1988) decided to look at the possibility of managing anxiety by reducing caffeine intake with anxious patients. He found that those who made reductions in their caffeine intake also had reductions in their anxiety, sleep disturbances, headaches, abdominal problems and their irritability. This brings into question the practice of providing coffee and tea freely in institutions when it may be prolonging the problems. Certainly it would be much better to discover if the anxiety problems of patients are related to caffeine rather than to treat them unnecessarily with drugs.

## BEHAVIOUR IN CAFFEINE EXPOSED ANIMALS

Anxiogenic-like effects have also been found in rats (File and Hyde, 1979; Baldwin et al. 1986, 1989) exposed to acute and chronic doses of

caffeine. Baldwin et al. (1986) tested rats both acutely and chronically over three weeks with two doses of caffeine. Two tests were used, the social interaction test and the elevated plus maze. The acute doses produced a decrease in interaction, and in rearing and a decrease in the entries to the open arms. The chronically treated rats showed no change in social interaction or in entries to the open arms. The acute doses of caffeine produced effects which suggested anxiogenesis but the chronic treatment appeared to cause tolerance in rats. Holtzman (1983) also found that rats developed tolerance to chronic caffeine's locomotor stimulating effects which was reversible when caffeine administration was stopped.

File et al. (1988) again found decreased social interaction and also reduced head-dipping, and less offensive aggressive behaviours in the home cage with an acute dose of 40 mg/kg. Motor activity was increased with 20 mg/kg. Chronic caffeine exposed rats did not show any differences from controls. This showed that the lower dose had a stimulant effect while the higher dose decreased exploratory behaviours. File and Hyde (1979) found an increase in motor activity with 20 mg/kg (ip) but a decrease in social interaction. Britton and Indyk (1990) tested rats in a novel open field and in the familiar home cage. The tests in the home cage showed that caffeine increased activity whereas in the open field, activity was lowered with doses of 5 and 10 mg/kg (ip). This suggests that it may be important to take the novelty of the testing apparatus into account when looking at the behavioural effects of caffeine.

Katims et al. (1983) and Snyder et al. (1981) reported that a biphasic effect was found with caffeine at different doses. At low doses, activity is reduced while at high doses activity is increased. Baldwin and File (1989) also found evidence for anxiogenic-like effects in rats with a reduction in social investigation with doses of 20 and 40 mg/kg caffeine.

Longterm anxiogenic effects have also been found in rats prenatally

or perinatally exposed to caffeine and this will be looked at in more depth in the next section.

## TERATOGENIC EFFECTS OF CAFFEINE

Pregnancy is a time of great vulnerability to the unborn child. This is the time of most rapid development and the foetus is in direct contact with many of the same chemicals as its mother. Unfortunately the effects on the fetus are more detrimental. The nervous system in particular is susceptible to damage during both pregnancy and the early part of life, particularly when breast-feeding. Therefore effects of drugs on the unborn child are important to understand. Caffeine is an appropriate drug to study since many mothers would not consider the risk that caffeine could be to their child because it is so generally accepted.

### CAFFEINE ABSORPTION AND DISTRIBUTION

Caffeine is readily absorbed from the gastro-intestinal tract with peak plasma levels occurring in 45 minutes to two hours. It is then rapidly distributed into body fluids with a volume of distribution of one litre per kilogram. After distribution, caffeine is eliminated from the body in urine when it has been metabolized by the liver. Caffeine is also eliminated via saliva, semen and breast milk, and is found in umbilical cord blood. Levels of caffeine in breast milk are only slightly lower than blood levels. The breast milk of a mother who has ingested 145.8 mg of caffeine, contains .82± .29mg/kilogram and the infant consuming this takes in about .027 to .203 mg of the drug (Eilenhorn and Barceloux, 1988).

The half life of caffeine in normal adults is 3 to 7.5 hours, but in pregnant mothers this time is lengthened, increasing until it is about 18 hours in the last three months of pregnancy (Sobotka, 1989) thereby causing longer exposure to the infant. In newborns the half life of caffeine is 82 hours (Aldridge et al., 1979). Most research suggests that caffeine should be avoided during pregnancy since the half life of caffeine in neonates can exceed 90 hours. As it is transmitted by the mother to the foetus, their blood level concentrations become similar (Gilliland and Bullock, 1984).

## PHYSICAL EFFECTS OF CAFFEINE ON HUMAN BABIES

There are many reports of complicated deliveries of babies born to mothers consuming 600 mg of caffeine or more. Weathersbee (1977) found only one such mother out of 16 in a study, had an uncomplicated delivery. The other 15 pregnancies ended in spontaneous abortion, still and premature births.This is rather a high rate when 78.4 per cent of women who consumed no caffeine had uncomplicated births. Fenster et al. (1991) found an increased risk for low birth weight for mothers consuming caffeine. Those consuming a large amount (over 300 mg) were at double the risk of those in the unexposed group. Medium consumers of caffeine (151-300 mg) had twice the risk of producing a baby with intrauterine growth retardation. Fathers may also be affecting pregnancy outcomes due to semen levels of caffeine, if they drink more than 600 mg of caffeine (Weathersbee, 1977).

## PHYSICAL EFFECTS IN ANIMALS WITH PRENATAL EXPOSURE

In spite of some results suggesting that caffeine may be a teratogen,

there is no real evidence for this in humans, Weathersbee's study used a very select sample which may not have been representative of the general population. If we turn to the animal literature there are many more studies suggesting that caffeine is a teratogen.

Gilbert and Pistey (1973) gave intraperitoneal injections to pregnant dams at doses ranging from four to 16 mg daily. Decreased birth weight and a greater number of resorptions occurred in groups given eight or 16 mg of caffeine. A decrease in litter size also occurred for these two groups. West et al. (1986) found reduced body weights in 50 and 70 mg/kg groups. Reduced body, liver and brain weight were found in offspring of rats with caffeine in their drinking water at a dose of 122 mg/kg/day by Groisser et al. (1982). During lactation the pups recovered their weight difference, so that at 30 days, they were similar to controls. Peruzzi et al. (1985) found no body weight change in perinatally caffeinated rats at doses of 27, 58 and 108 mg/kg.

Among the physical abnormalities that have been found with prenatal caffeine exposure are visceral and skeletal abnormalities, such as irregular and incomplete ossification of the skeleton and absence of supraoccipital bone (Fujii and Nishimura, 1972; Palm et al., 1978), hydrocephaly, oesophageal defect, edema, tail defects (Fujii and Nishimura, 1972), delays in eye opening, incisor eruption and vaginal opening (West et al, 1986), cleft palate, lighter brains, liver and lungs (Palm et al., 1978). The doses given to the pregnant dams and the route of administration varied in these studies, but some physical damage to foetal rats is obvious. Fujii and Nishimura looked at caffeine effects at different stages of pregnancy and found damage seems to be confined to caffeine exposure at certain times during pregnancy. Most of the lethal and teratogenic damage occurred in the middle of pregnancy in their rats whereas edema was characteristic of the last part of pregnancy.

Brain abnormalities have been examined by a number of researchers. Nakamoto et al. (1986) have proposed that even when a small amount of caffeine is ingested by pregnant dams, this will affect the foetal brain because this is the time of greatest growth and development. Therefore it is more susceptible to influences such as caffeine. The cholesterol contents of the medulla oblongata, striatum, midbrain and hippocampus were lower than controls, protein content was lower in the medulla oblongata and the DNA in the striatum was also lower than controls.

Yazdani et al. (1990) reported that different effects are displayed by different amounts of maternal caffeine ingestion. Foetal brain weights were heavier when the mothers ingested 2 mg/100 grams of bodyweight than when they ingested 0.5mg/100 grams. DNA concentrations were increased in the 0.5 mg exposed group whereas in the 2 mg exposed group the protein concentration was higher.

Decreases in cyclic AMP were found by both Concannon et al. (1983) and Peruzzi et al. (1985). Even ten days after withdrawal from caffeine, the whole-brain levels were lower in the Concannon et al. study, whereas cerebellar cyclic GMP was elevated. However, this study was confined to postnatal caffeine exposure.

The physical effects of prenatal caffeine exposure are numerous although not consistent, and may not be as important or common as behavioural changes. Many researchers have not found any evidence of physical abnormalities in the offspring of animals (Palm et al, 1978) in a similar fashion to what characterises human caffeine exposure. It is reasonable to expect that with caffeine effects on the brain, changes in behaviour will occur. This may be the more fruitful area to pursue.

## BEHAVIOURAL EFFECTS OF CAFFEINE IN ANIMALS

The behavioural effects of prenatal and perinatal caffeine have been mainly studied in rats and mice. It is more difficult to study behavioural or physical effects in humans due to ethical cosiderations. Retrospective studies are difficult to carry out because the amount of caffeine ingested is hard to determine when relying on self reports. People do not like to always admit that they have consumed a lot of caffeine if some adverse effects on their child has occurred. Therefore most research has focused on the rat. Considerable interest has arisen over the issue of whether or not pre/perinatal caffeine has longterm or permanent effects on offspring. The longer the period after birth that testing is carried out, the more likely that effects are permanent. Many researchers have tested rats at various time periods after birth.

The research can be divided into areas of prenatal and perinatal caffeine exposure effects. A few studies have looked at postnatal exposure but most have focused on the other two areas combined. Within each division, further differences can be found in regard to the amount or dose levels given to the mothers. Other differences between studies which may confuse things are the route of administration of caffeine and the behavioural testing methods. These differences between the studies do make comparisons difficult but there are many trends that do emerge from them.

One aspect of the experimental designs which makes the study more realistic for humans is the method chosen for administering caffeine. Studies which have provided caffeine in the female rats' drinking water are more relevant than those in which the drug has been injected daily. Palm et al. (1978) showed that the outcome on the offspring was different if the

caffeine was given in the drinking water than if it was injected in one dose every day. Physical abnormalities seem to be more prevalent when it is injected than when provided ad libitum in the drinking water.

Crossfostering the offspring after birth is another practice which is often employed. This allows a much more conclusive study since it eliminates environmental effects from the mothers' altered behaviour because of the caffeine ingestion. Studies which have fostered the young generally support the idea that behavioural changes result from caffeine, and not just altered maternal behaviour.

An inconsistency in the literature does exist regarding the dose levels of caffeine which are given to the mothers. Usually a low and a high dose as well as a placebo are used to allow for dose-response interaction, but the amount of caffeine in these two levels varies greatly. Sobotka (1989) reviewed a number of studies and found that the low dose ranged from 5 mg/kg/day to 60 mg/kg/day. The high dose ranged from 35 to 180 mg/kg/day. As well as this problem, Sobotka also drew attention to the small number of studies that measure the blood levels of caffeine in the dams during exposure. This procedure would provide more accurate calculations of the amount of caffeine that is actually circulating in the rats.

The method of testing used to look at the behaviour of rats has also been quite varied. Some of the tests employed are the open field test (Hughes and Beveridge, 1986 ; Sobotka et al., 1979; Glavin and Krueger, 1985), emergence apparatus (Hughes and Beveridge, 1986), social interaction (File and Hyde, 1979; Holloway and Thor, 1984), elevated plus maze, passive avoidance task (Sobotka et al. 1974; File, 1987), photocell activity cages (West et al. 1986), running wheel activity (Butcher et al. 1984), righting reflex, swimming, chimney test (Peruzzi et al., 1985), holeboard test (File et al, 1988), and spontaneous alternation (Sinton, Valatx and Jouvet, 1981). Within each of these different tests, the procedures also vary. The open

field test has been conducted in many different ways which has led to much criticism (Walsh and Cummins, 1976). Most tests however are used to measure activity or emotionality and a fair amount of consistency is found in the results.

## PRENATAL CAFFEINE EFFECTS

There are a great number of studies which have looked at the effects on the offspring of maternal caffeine exposure during pregnancy alone. Hughes and Beveridge (1986) tested their rats at various ages (61, 145 and 188 days) in an open field after dams had been injected during gestation with doses of 10, 20, or 40 mg/kg or with distilled water. When younger, rats tended to have increased locomotor activity and male rats exposed to 40 mg/kg walked significantly more than those exposed to 20 mg/kg or controls. As they became older, rats groomed more and stayed in the corners, suggesting a more emotional/fearful reaction. The pregnant dams in this research were injected intraperitoneally with caffeine. Later, Hughes and Beveridge (1987,1990) changed their method of caffeine administration by providing it in the drinking water. Three ages of testing were again used in the 1987 study, 73, 117, and 171 days. The doses given to the female rats were 24 or 44 mg/kg and tap water alone for the controls. Decreased locomotor activity was found at the high dose for all ages and females' defecation decreased while more males failed to emerge with the high dose. These results are suggestive of greater emotional reactions with the high dose of caffeine.

Biphasic effects have been found with caffeine doses. Often a low dose will increase activity and decrease emotionality while a high dose decreases activity and probably increases emotionality. Further evidence for

this was heavier adrenal glands in females exposed to 36 mg/kg/day of caffeine than controls. Adrenal glands are known to grow in rats exposed to stressful situations (Hughes and Beveridge, 1990). Doses used were 28 and 36 mg/kg. Lombardelli et al. (1984) also found a biphasic effect in rats. This occurred for both perinatally exposed rats and controls. Activity increased with a maximum peak at 30 mg/kg caffeine exposure and then decreased with higher doses of 60 mg/kg. The perinatal caffeine rats had lower activity at both points than controls, so that the main effect was depressed activity in the perinatally treated rats compared with controls. Butcher et al. (1984) also found increases in running wheel activity with low doses of prenatal caffeine and decreases with the high dose. This effect seems to be specific for prenatal and perinatal caffeine treatment. With acute postnatal treatment the opposite is found. Snyder et al. (1981) have reported that caffeine reduced activity at lower doses of five and ten μmol/kg while stimulating activity at higher doses of 30 and 100 μmol/kg. The effects found with prenatal exposure must be due to longterm changes in the brain rather than an effect of withdrawal or presence of caffeine in the brain as this occurs long after caffeine exposure has stopped.

Glavin and Krueger (1985) found no significant differences between controls and prenatal caffeine-exposed rats in open field ambulation or defecation, but they did find that rats who were exposed to the high dose (0.5 mg/ml) had more (and bigger) gastric ulcers than did controls. This agrees with the results of Hughes and Beveridge suggesting that increased emotionality (anxiety/fear) occurred.

Sinton, Valatx and Jouvet (1981) have also found evidence for behavioural teratogenic effects in mice. An increase in passive avoidance latencies at doses of 60, 80 and 100 mg/kg/day was found along with an increase in open field activity in female mice only. West et al. (1986) also

found an increase in passive avoidance with a prenatal caffeine dose of 50 mg/kg/day. At lower doses, 5 and 25 mg/kg/day, passive avoidance was decreased.

Concannon et al. (1983) gave rats caffeine prenatally (14.24 mg/kg/day average), postnatally (52.23 mg/kg/day) or during both times (39.41 mg/kg/day) in the dams drinking water. The postnatal caffeine group has lowered activity at 15 days and the perinatal and postnatal groups both showed lowered activity from day 20. Postnatal caffeine therefore seems important in reducing activity. At 25 days the postnatal caffeine treatment stopped and by 35 days only the prenatal caffeine group had reduced activity. This indicates that prenatal caffeine gave longerlasting behavioural effects than postnatal caffeine.

## PERINATAL CAFFEINE EFFECTS

Perinatal caffeine effects have not been tested very often, but a few studies do exist. They give a better indication of what might really happen since most human mothers would continue to drink caffeine while breast-feeding if they had done so throughout pregnancy.

Sobotka, Spaid and Brodie (1979) exposed rats to caffeine in their drinking water during pregnancy. The exposure doses to the offspring were 23, 49, and 92 mg/kg/day during gestation and 37, 75, and 138 mg/kg/day during lactation. Perinatal-caffeine exposed rats at all doses had increased rearing in an open field. Ambulation was also increased in the low and middle dose groups. Stress responses were not affected.

Peruzzi et al. (1985) used caffeine dose levels of 0, 27, 58, and 108 mg/kg/day during gestation and 0, 49, 103, and 188 mg/kg/day during lactation. Behavioural tests revealed that start latencies were increased in all caffeine treatments, the number of squares entered were reduced for all but

the high dose, sniffing and rearing was decreased. Only the sniffing and rearing measures were significantly different from controls. Effects were strongest for the lowest doses and as the doses increased they became more like controls. The rats in this study were tested before they were withdrawn from the caffeine treatment and this can confound the results. Sobotka et al (1979) mentioned above tested the rats after they were weaned so their results are not comparable with Peruzzi et al. Concannon et al. (1983) measured the rats' behaviour at several time periods and did find hypoactivity in preweaned rats which is consistent with the Peruzzi et al. study.

Hughes and Beveridge (1991) have completed a study directly relevant to research which will be presented in this thesis, as the rats used in this study were siblings of those tested by Hughes and Beveridge. The investigation by Hughes and Beveridge was similar to the Concannon et al. (1983) study. Rats were given caffeine during gestation, lactation or both. Doses of 0, 26 and 45 mg/kg/day were provided for dams in their drinking water during gestation and in lactation the doses were 0, 25, and 35 mg/kg/day. The difference between the gestational and lactational doses were due to differences in the amount of water ingested during the two time periods. The rats were tested at various ages. These were one, two, four and six months after birth.

Rats in the gestation group only were affected in the following way:

1) Males with the low dose walked less than controls or the high dose.
2) Less rearing occurred for two and four month old rats at the high dose.
3) Ambulation was greater for males at both caffeine doses.

4) Rats exposed to the low dose had greater latencies or failed to emerge more often.

Rats in the lactation group only were affected in the following way:

1) Rats exposed to the low dose reared less often
2) Less ambulation occurred in the high dose male group.
3) Males in the low dose group defecated more than those in the high group

More behaviourally significant results were obtained for the rats that were perinatally exposed to caffeine. This suggests that caffeine exposure during both gestation and lactation has greater effects on the offspring than during one period alone.

1) Rats exposed to low or high doses had higher walking than controls
2) Rearing was lower with both doses than with controls.
3) Ambulation was lower in both dose treatments.
4) Greater defecation occurred for both dose exposed rat group but only when tested at six months of age.
5) A greater number of rats in the two caffeine dose groups took longer to emerge or failed to emerge than the controls.

A significant finding in this last group was the loss of male only effects which were evident in the lactation only and gestation only conditions. Activity was reduced for both dose levels of caffeine in the perinatal group in every case whereas the the prenatal and postnatal groups were usually affected by one dose only. This suggests a greater additive effect of caffeine when exposure occurs in both gestation and lactation.

The measures suggest that decreased activity and increased emotionality, may characterize the effect caffeine has on these rats.

## EXPLANATIONS FOR CAFFEINE'S EFFECTS

Reasons for the behavioural effects which have emerged in the data have been sought by many investigators in an attempt to discover how caffeine influences behaviour and the brain. The behavioural changes appear to be long lasting. They last at least eight months as found by Hughes and Beveridge (1990), so it is important to understand implications for human beings.

There have been many theories proposed to account for caffeine's effects, some accounting for the stimulant properties of caffeine and others looking at the anxiogenic effects of caffeine. The main ones involve phosphodiesterase action, dopamine system interaction for the stimulatory effects and, for the anxiogenic effects, increased catecholamine activity, benzodiazepine receptor blockade and adenosine receptor blockade.

### PHOSPHODIESTERASE INHIBITION

A mechanism proposed to explain caffeine's stimulant effects is the inhibition of phosphodiesterase. This enzyme is responsible for changing the shape of the protein on the neuronal membrane gate. If phosphodiesterase is inhibited, cyclic AMP accumulates which results in the ion gates staying open. Caffeine has been found to inhibit phosphodiesterase so that this situation does occur and this has been the proposed mechanism responsible for caffeine's stimulant and wakefulness effects. Apart from phosphodiesterase, the neurotransmitter seratonin can prolong cyclic AMP which may also show an increase with caffeine. Drugs

such as buspirone, which is an anti anxiety drug, may perhaps work through this mechanism.

This phosphodiesterase hypothesis has problems. Peruzzi et al. (1985) and Concannon et al. (1983) found that cyclic AMP levels were actually decreased rather than increased with perinatal caffeine treatment which argues against this mechanism being involved. The concentrations of caffeine that would be needed for phosphodiesterase inhibition to have any effect are far greater than the amount of caffeine that is found in the brain at effective doses (Smellie et al., 1979). Another problem is that other phosphodiesterase inhibitors do not show the same behavioural effects as caffeine and some are central depressants (Beer et al., 1972).

## DOPAMINE

The involvement of dopamine in the effects of caffeine has been suggested. Boehne and Ciarenello (1981) found that activity was influenced by dopamine receptor concentrations. Sinton, Valatx and Jouvet (1981) have suggested that systems related to anterior forebrain dopamine systems may be changed by caffeine given during gestation. They report one study in which caffeine during gestation resulted in a decrease in dopamine in the locus coeruleus. However, Concannon et al. (1983) found no effect on dopamine levels with caffeine exposure pre and postnatally. The relationship of dopamine is unclear.

## CATECHOLAMINE ACTIVITY

Norepinephrine (NE) is known to increase with caffeine administration (Charney et al., 1984; Charney, Heninger, and Jatlow, 1985).

Robertson et al. (1978) found increased levels of NE in the plasma and urine of humans. Animal studies have also found an interaction between caffeine and NE function (Corrodi et al., 1972).

Norepinephrine effects peripheral autonomic synapses (Sawyer, Julia, and Turin, 1982). In many anxious patients, peripheral NE hyperactivity is displayed. These are the somatic symptoms of anxiety including palpitations, stomach butterflies, tremulousness, and hyperventilation. The relationship between caffeine's anxiogenic properties and these symptoms has been explained as NE increase. In particular, panic anxiety has been associated with this hypothesis due to the symptoms experienced in a panic attack (Charney et al., 1985). Beta blockers have been utilized medically in light of this peripheral NE proposal because they decrease NE activity possibly by both central and peripheral blockade (Hayes and Schulz, 1987).

However, a problem with this hypothesis exists because many studies have failed to find increased levels of plasma MHPG, the major metabolite of NE (Charney et al., 1985; 1984; Concannon et al., 1983; Uhde et al., 1984). This could be explained by the fact that at low doses of caffeine, MHPG levels do not increase and at higher doses only a twenty per cent increase of MHPG may occur (Charney et al., 1984). If small increases occur in MHPG in regional brain NE turnover, this may not be reflected in total brain or plasma MHPG levels.

One brain region which is implicated in regulating anxiety, is the locus coeruleus, a nucleus in the brainstem. This region contains 70 per cent of the brains noradrenergic neurons and projects to the limbic system, cerebral and cerebellar cortices (Hayes and Schulz, 1987). This gives support to the role NE may play in anxiety and in caffeine's effects in anxiety. What is not really understood however, is the way that beta blockers exert their

action. They may be blocking only the peripheral beta sites or they may also act centrally. Most studies do suggest that they act on the periphery (Hughes, 1981), but they may act centrally as well by inhibiting the NE system via the locus coeruleus or indirectly by peripheral blockade and feedback to the brain. Direct blocking of beta-adrenergic impulses may also occur (Noyes, 1982).

The importance of NE in caffeine's anxiogenic effects is still unclear. The lack of positive increases in MHPG has made the involvement more difficult to find which has led many researchers to regard it as less likely than other brain systems.

## BENZODIAZEPINE RECEPTORS

Benzodiazepine receptor antagonism is a popular hypothesis to explain caffeine's behavioural effects. The benzodiazepine drugs are very commonly used to alleviate anxiety. These benzodiazepine drugs act on the benzodiazepine -receptor- GABA complex (Uhde, Tancer and Gurguis, 1990). The effects that caffeine have on the benzodiazepine receptor have been very mixed. Caffeine was found to antagonize diazepam's central effects which suggests that it is blocking the benzodiazepine receptor. Caffeine was found to increase (Wu and Coffin, 1985; Boulenger et al., 1983)the numbers of benzodiazepine receptors but Marangos, Boulenger and Patel (1984) found no increase in benzodiazepine receptors from day 16 to 23 of caffeine treatment while they were increased up to day 16. Even if the benzodiazepine receptor is not antagonized by caffeine, benzodiazepine action may be antagonized at the adenosine receptors or an unknown site (Wu and Coffin, 1985), or it may even occur at both sites. Phillis and Wu (1981) have shown that benzodiazepines block adenosine reuptake therefore allowing endogenous adenosine to accumulate extracellularly. The

benzodiazepines' sedative and muscle relaxant effect was suggested as being due to the increase in extracellular adenosine (Clanachan and Marshall, 1980). This last possibility can not be ruled out, according to Bruns et al. (1983).

Wu and Coffin looked at the withdrawal effects from chronic caffeine at the benzodiazepine receptor site and the adenosine receptor site. A 30.9 per cent increase in diazepam binding and a 120 per cent increase for the adenosine agonist PIA was found. This may indicate a lower affinity for benzodiazepine receptors than adenosine receptors. Wu and Coffin's conclusions were that both the benzodiazepine and adenosine receptor sites may be affected by chronic caffeine and therefore, both may be responsible for the behavioural effects found.

Snyder et al. (1981) decided that caffeine's behavioural effects are unlikely to be mediated at the benzodiazepine receptor site because the "methylxanthines are 100 times more potent at the adenosine than benzodiazepine receptors and no correlation exists between behavioural potencies and effects at benzodiazepine receptors." Marangos et al. (1981) also found the benzodiazepine receptor to be an unlikely site for caffeine's behavioural effects because the levels of caffeine required to affect them are found at convulsant doses of caffeine (Marangos et al., 1981). So, most evidence points to the adenosine receptor site for the behavioural effects. It may be that benzodiazepines do help to potentiate the adenosine as Wu and Coffin felt or it may be that caffeine affects adenosine alone.

Commissaris et al. (1990) reported that benzodiazepines do not appear to exert their effects by inhibiting adenosine reuptake which has been suggested since two adenosine agonists, *l*-PIA and NECA both failed to effect a behavioural test which is sensitive to benzodiazepines. If increased adenosine was occurring with the use of benzodiazepines then one would

expect these analogs to have a similar effect.

The interaction between benzodiazepine and adenosine appears to be small. There are no direct receptor interactions. Adenosine and benzodiazepines do not seem to affect each other's receptor sites. There may be some influences on the output of adenosine by benzodiazepine. Therefore, adenosine appears to be more important in caffeine's effects than the benzodiazepine receptors.

## ADENOSINE RECEPTORS

The strongest explanation for caffeine's anxiogenic effects involves adenosine receptors.

Adenosine is a purine nucleoside. It is present in many brain areas as well as existing in the periphery. Adenosine is best described as having a neuromodulatory role although it does have neurotransmitter-like properties but does not fill all the criteria for a neurotransmitter. As a neuromodulator it affects neurotransmitters mainly in an inhibitory fashion. Adenosine has been recognised as playing an important role in controlling excitability, having a sedative effect. Katims, Annau and Snyder (1983) found that sedation occurred with direct administration of adenosine to the brain. Locomotor activity is also reduced by adenosine and this effect is biphasic, which may possibly implicate an involvement of the differential effects of the A1 and A2 receptors (Williams, 1987).

There are three adenosine receptor sites which have been identified. They are called the A1, A2 and P receptors. At present, the P receptor is not very well understood. Both the A1 and A2 have been studied more extensively and are better understood although there is still a lot to learn. It

is known that A1 and A2 receptors are situated extracellularly and have effects on adenylate cyclase, an enzyme which is responsible for production of cyclic AMP. A1 receptors inhibit adenylate cyclase whereas A2 receptors stimulate it. This influence on adenylate cyclase may be responsible for the sedating role of adenosine. A1 will cause more stimulation by inhibiting adenylate cyclase which will produce cyclic AMP causing the ion gates to remain open, whereas the A2 receptors increase the adenylate cyclase therefore having the opposite effect and sedating the individual. This relationship is complex however, because both A1 and A2 agonists cause sedatory effects.

Caffeine appears to antagonize both A1 and A2 receptors. Following caffeine administration, adenylate cyclase is sensitized to adenosine according to Stiles (1986). Furthermore the caffeine dose required to affect adenosine receptors are well within the stimulant dose range in rats (Snyder et al., 1981). Chronic treatment with caffeine has also been found to cause an increase in adenosine receptor numbers (Boulenger et al., 1983).

Marangos, Boulenger and Patel, (1984) have examined the relationship between adenosine and caffeine consumption, in particular looking at the receptor upregulation and distribution in the brain in mice, on a daily basis, exposed perinatally to chronic caffeine. There animals were given caffeine doses equivalent to 4 to 5 cups of coffee per day in humans when surface area and metabolic differences are taken into account.

Increased numbers of receptors were found at day 11 but not earlier and the mice were weaned at day 14. Therefore, they concluded that caffeine during lactation was sufficient to increase the number of adenosine receptors. No postweaning/ post caffeine tests were made. Since all measurements were during caffeine exposure there was no evidence of longterm changes to adenosine receptors, although this has been suggested as the reason for the longterm behavioural changes. The altered adenosine

receptors found in this study were certainly consistent with studies showing behavioural changes with perinatal treatment, even though this study did not measure behavioural changes.

The adenosine uptake site was not affected by the chronic caffeine administration which confirmed the belief that the uptake site and receptors are separate and have different regulation. This lack of uptake receptor effects may also provide more contrary evidence for benzodiazepines acting via the adenosine system. Areas of the brain that showed adenosine receptor increases were the cerebellum and brainstem to the greatest extent. The cerebral cortex and the thalamus were not as easily influenced, no significant differences in these regions were found until day 23. Surprisingly, the adenosine receptors in the hippocampus where receptors are normally very dense, were not increased.

Snyder et al. (1981) took the relationship of caffeine with adenosine one step further and investigated whether behavioural effects of xanthines occurred with increased adenosine receptors at acute doses of caffeine. They found that increased locomotor activity correlated with the increases in adenosine receptors labelled with $^{3}H$ - CHA suggesting that the stimulatory effects of caffeine are related to adenosine upregulation.

Adenosine analogs can be useful for determining if the effects of adenosine are the same as when levels have been increased in the way suggested with caffeine ingestion. Snyder and Sklar (1984) used two of these analogs, CHA and PIA. Both are specific A1 agonists. At low doses, 0.1 µmol/kg (ip) *l*-PIA was found to reduce locomotor activity. Lower doses than 0.1 µmol/kg (.005 to .001 mg/kg) lead to stimulation of locomotor activity (Katims et al., 1983). Even when doses are 500 times greater than 0.1 mg/kg, mice remain awake and lethal effects do not appear to exist, even at 800 µmol/kg (Snyder et al., 1981). Dunwiddie (1985) points out the

importance of intracerebroventricular injections to get a clear picture of effective doses. This is because of the 0.02 to 0.05 mg/kg (ip) range he reported as exerting depressant effects, only 10nM or less entered the brain.

If depressant doses of L-PIA and caffeine are given together the effect is a 300 per cent stimulation. This is probably because some sites have high affinity for L-PIA and these characterise the stimulatory effects. Other sites have lower affinity and display depressant effects. Xanthines may block these lower affinity sites for L-PIA and thus the stimulant effects are brought out by L-PIA at the high affinity sites (Snyder and Sklar, 1984).

Boulenger et al. (1987) found some indirect evidence for adenosine being involved in the anxiogenic effects of caffeine in humans. Caffeine levels which produced anxiety (720 mg) are in the range known to compete with the binding of various ligands to the adenosine receptors in the human brain. They failed to find elevated plasma adenosine levels but this may be due to the fast reuptake of adenosine when displaced from brain receptors.

Adenosine upregulation is certainly the most likely theory to explain the anxiogenic effects of caffeine. However, there are problems, in particular, the fact that reduced activity (found with perinatal caffeine) is consistent with increased adenosine, whose major function appears to be sedative. Thus, the increased emotionality/anxiety would not be expected. More needs to be known about adenosine's actions and the interaction with caffeine. It may be that different receptors are involved or that adenosine interacts with other neurotransmitter systems.

## HYPOTHESES IN THIS STUDY

Perinatally caffeine treated rats will have different behaviour to rats who have not been exposed to caffeine. As demonstrated earlier (Hughes and Beveridge, 1991), this will be shown as decreased locomotor activity measured by ambulation, walking, rearing and amount of time spent still. Increased emotional effects may be evident by increased defecation, higher corner occupancy and a longer emergence latency from a dark to an illuminated area.

The use of drugs, mainly antianxiety, which have affinity for specific brain receptors will give differential effects for the control, low and high perinatal caffeine groups. This will assist in discovering which brain mechanisms are involved in the behavioural effects of perinatal caffeine exposure.

Administration of caffeine in acute doses will show differences between the groups as there may be tolerance effects which are longlasting or a permanent change in the brain which may cause a difference in caffeine's influences on caffeine-exposed rats.

## RATIONALE

This experiment is a continuation of research by Hughes and Beveridge (1991) on the behavioural effects of perinatal caffeine exposure in rats. They found differences between the groups; the caffeine groups showed reduced activity and signs of increased emotionality when observed in the open field. The inference was that a permanent change in the brains

of these animals may have occurred. It was suggested that a likely reason for this was an upregulation of adenosine receptors, based on findings by other researchers that have already been mentioned.

This study is a continuation of the original, in which untested siblings were further investigated by observing the effects of specific drugs in order to gain a better understanding of perinatal caffeine effects in rats. If the mechanisms responsible for behavioural changes caused by caffeine could be discovered, this would assist in the reversal of problems which may exist in humans exposed to caffeine during pregnancy and while breast-feeding.

The behavioural test employed was similar to that used by Hughes and Beveridge. This seemed the most logical way to conduct the experiments, so that the findings could be consistent and related to the original study. The open field arena is a useful test method for observing behavioural effects. While it is a simple test which gives results fairly quickly, it has been criticized for its simplicity and because there is no standard open field apparatus. The arena can be any shape (usually circular or square) and any size. Other inconsistencies exist in regard to the time sampling of observations, the definition of the measures used and in the lighting and noise levels employed (Walsh and Cummins, 1976). However, it is a test which allows the animal relatively free choice in its movements which is important when measuring behaviour. The less disturbance of the animal the more natural its behaviour will be. It is a useful test for acquiring some idea of the animal's behaviour from which further, perhaps more rigorous, tests can be employed.

The behavioural measures chosen were indices of activity and fear. Walking and ambulation are usually related measures of activity with the amount of time spent still showing an expected inverse relationship.

Rearing also shows the level of excitability and has been described as possibly being more of an escape response, whereas walking and ambulation may be exploratory in function. Grooming is usually negatively related to high activity. Higher grooming is regarded as indicative of increased fear, acting as a displacement behaviour. Defecation is probably the most reliable measure of emotionality. Immobility and freezing are also signs of stress, and corner occupancy, rather than centre occupancy is an index of timidity (Archer, 1973).

The drugs employed in this study were caffeine, diazepam (a benzodiazepine anti-anxiety drug), Chlorohexyladenosine (CHA, an adenosine A1 agonist) and oxprenolol (a Beta blocker). This type of study has not been caffeine effects and previously carried out.

Baldwin and File (1989) have looked at behavioural effects of acute caffeine and the possible brain mechanisms influencing this. They used similar drugs influencing the same receptor populations in the brain but they measured social interaction effects. There study is not comparable to this one because of the known differences between acute doses of caffeine and chronic perinatal exposure.

Rats are suitable animals for this investigation because of their availability and the past work done with them. Rapid results are obtainable and most importantly, the data is not retrospective and reliant on self reports. The ethical problems of studying humans in this light are also obvious and virtually impossible to do at this time.

# CHAPTER TWO
# METHODS AND MATERIALS

## ANIMALS

Subjects were 72 Wistar rats, exposed to caffeine perinatally through their mothers' drinking water during gestation and while lactating. All rats had been bred by Hughes and Beveridge (1991) for another purpose. The particular subjects used in this study had not been tested previously. There were three different condition groups: the mothers' of one group received only tapwater during pregnancy and lactation. Their offspring comprised the control group. A second group received 26 mg/kg/day caffeine added to their mother's drinking water during gestation, and 25 mg/kg/day during lactation (low dose). The third group received a high dose of caffeine via their mothers' drinking water which contained 45 mg/kg/day during gestation and 35 mg/kg/day during lactation. The rats were also crossfostered during this process to control for effects by the dams. All rats were born at the same time and tested approximately 9 months after birth.

## HOUSING

The rats were housed in groups of three in single sex cages. Food and water was made available to them at all times and they were kept in a 12-hr light: 12 hr dark cycle, and tested during the dark cycle. The rats were sprayed with nontoxic dye for identification purposes.

## DRUG TREATMENT

Injections were given intraperitoneally , in a volume of 1 ml/kg. Each rat received five injections over the course of the experiment. Injections were given at least one week apart, and the order of drug

administration was varied.

The drugs used were saline (isotonic solution), Diazepam at two doses (1 and 2 mg/kg bodyweight), caffeine (10 and 20 mg/kg bodyweight) Chlorohexyladenosine (CHA, 0.05 and 0.1 mg/kg bodyweight) and oxprenolol at two doses (10 and 20 mg/kg bodyweight). After injection of the drug, the rat was left for 30 minutes before testing.

APPARATUS

A square open field arena was used, measuring 60cm x 60 cm and with 30 cm high walls. The black perspex floor was divided into 16 squares which were numbered accordingly. A lamp with a circular fluorescent tube was placed above the open field to provide equal light over the whole arena.

An emergence apparatus was also used, consisting of a small dark room, 20 cm x 15 cm with 20 cm high walls, separated from a larger box, 50 cm x 40 cm with 20 cm high walls, lit by two fluorescent tubes in the floor. There was a sliding door in between the two rooms which allowed access to each when opened.

White noise was used to mask outside noises and was kept at a level of 58dB for the entire time that the rats were in the testing room. A handheld beeper was used, which sounded every five seconds through an earplug which only the experimenter could hear. A stopwatch was also used, to record latency to emerge from the emergence apparatus.

PROCEDURE

The rats were placed into two independent groups, split equally to contain the same number of each condition group and sex. This formed two separate experiments.

Experiment one consisted of testing the 36 rats with saline, the two

doses of diazepam, and of caffeine. A repeated measures design was used so that every rat experienced each different dose over a five week period but in a different order. A week intervened between each test to ensure that the drug effects had dissippated and also to reduce familiarity of the test procedure. The experiment was conducted using a single blind testing procedure. The treatment group for each rat was only revealed after all testing had been completed.

For ease of testing, all three rats in each cage were tested in succession. Therefore rats in the same cage were always tested on the same day, usually within the same hour.

Experiment two was conducted in the same manner but different drugs were used. These were saline, chlorohexyladenosine at low and high dose and oxprenolol at low and high dose.

Once injected, rats were left for 30 minutes in their cage before being placed by hand into the centre of the open field. They were observed for 5 minutes at 5 second intervals, when their behaviour was noted. The number of the square they were occupying was also written down. After five minutes the rat was removed from the open field, the number of faecal boli were counted and it was placed into the small dark box of the emergence apparatus. The sliding door was then opened and a stopwatch started. The time for emergence, defined as the complete entry into the lit area of the emergence box, was noted. If the rat failed to emerge within five minutes, this was recorded as "failed to emerge." The rat was then placed back into its cage and left for at least a week before retesting with a different drug or dose.

After each rat had completed its test, the open field and the emergence apparatus were washed down with soapy water to prevent the possibility of olfactory cues influencing later rats' behaviour.

DEFINITION OF MEASURES

1. Ambulation was measured by noting the square a rat occupied. Both back feet had to be in the square.
2. Corner Occupancy involved being in one of the four corner squares.
3. Centre Occupancy involved occupying one or more of the four centre squares.
4. Defecation was measured by counting the number of boli .
5. Emergence latencies comprised fully moving from the dark to the lit area was timed.
6. Grooming involved licking, washing, or with front paws over face.
7. Rearing was defined as standing up on hind legs either in mid air or against a wall.
8. Still behaviour involved not moving but they could be sniffing and/or moving their head.
9. Walking involved locomotion while observed.

DATA ANALYSIS

Three way ANOVAs were performed for each drug (dose x sex x perinatal caffeine group) for the measures of ambulation, centre occupancy, corner occupancy, defecation, emergence, grooming, still, rearing and walking.

One way ANOVAs were also performed to further analyse any significant interactions.

# CHAPTER THREE
# RESULTS

## EXPERIMENT ONE

Effects on behavioural measures, with administration of saline, diazepam and caffeine doses after perinatal caffeine exposure, for each sex are outlined in Figures 1-9. These follow after the written results for experiment one.

### DIAZEPAM

#### AMBULATION

There was an overall sex effect, $F(1,29) = 13.062$, $p < .005$. This was due to higher ambulation by females, (females = 38.574 ± 1.288, males = 30.255 ± 1.885).

A dose effect was also present, $F(2,58) = 36.630$, $p < .001$. Lowered ambulation occurred with the high dose of diazepam compared with the saline and low dose: the mean for the saline group was 39.8 ± 1.933, the low dose, 39.114 ± 1.993 and for the high dose, 24.686 ± 1.364.

These two effects further interacted to produce a sex x dose interaction, $F(2,58) = 3.187$, $p < .05$. As with the overall effects, a dose effect for males, $F(2,58) = 11.339$, $p < .0001$, showed lower ambulation with the high dose of diazepam than with the saline or low dose of diazepam. The saline dose mean was 35.824 ± 2.861, for the low dose it was 32.118 ± 2.811 and for the high dose, 22.824 ± 1.85.

A dose effect for females also existed, $F(2,58) = 29.139$, $p < .0001$, the high dose of diazepam resulting in lower ambulation than saline or the low dose. Means were 43.556 ± 2.356 for saline, 45.722 ± 1.49 for the low dose and 26.444

± 1.956 for the high dose. F(2,17)= 33.667, p,.0001.

The interaction was due to no sex effect for the high dose, but males had lower ambulation than the females for the saline dose F(1,78)= 5.323, p<.05 and the low dose of diazepam, F(1,78)= 17.546, p< .0001. Means for the saline dose were 35.824 ± 2.861 for males, 43.556 ± 2.356 for females. For the low dose, males scored 32.118 ± 2.811 and females, 45.722 ± 1.49.

CENTRE OCCUPANCY

An overall dose effect was present, F(2,58)= 8.221, p< .001. There was decreased centre occupancy with the high dose compared with saline and the low dose ( saline mean = 5.457 ± .737, the low dose = 6.771 ± .89 and the high dose = 3.114 ± .508).

A perinatal caffeine effect also occurred, F(2,29)= 4.438, p< .05, the control group having lower centre occupancy than the low perinatal caffeine group and the high perinatal caffeine group, ( the control group mean = 3.303 ± .475, low perinatal caffeine group = 5 ± .582 and the high perinatal caffeine group = 6.889 ± 1.164).

CORNER OCCUPANCY

There was an overall dose effect, F(2,58)= 3.236, p< .05, due to lower corner occupancy with the low dose than with the high dose, F(2,34)= 3.518, p< .05. Mean scores were 29.457 ± 1.556 for saline, 25.429 ± 1.546 for the low dose and 30.743 ± 2.005 for the high dose.

DEFECATION

A sex effect, F(1,29)= 34.271, p< .05, was found to be due to males defecating more than females, (males = 3.431 ± .48 and females = .389 ± .164).

There was a dose effect, $F(2,58)= 7.480$, $p< .005$, which was due to lowered defecation with the high dose of diazepam ($M=.886 \pm .352$) compared with saline ($M= 2.286 \pm .456$) and the low dose ($M=2.429 \pm .508$).

A sex x dose interaction was found, $F(2,58)= 3.887$, $p< .05$, which was due to a dose effect for males only, $F(2,58)= 10.269$, $p< .0001$. There was lower defecation with the high dose of diazepam compared with saline. The mean for the saline dose was $3.824 \pm .649$ and for the high dose, $1.824 \pm .66$.

EMERGENCE

An overall sex effect, $F(1,29)=5.412$, $p< .05$, revealed that females had shorter emergence latencies than males($113.3 \pm 21.905$ for females and $189.49 \pm 25.568$ for males).

There was a dose effect, $F(2,58)= 4.464$, $p<.02$, due to shorter latencies to emerge with the low dose ( $125.4 \pm 22.853$) compared with the high dose ($185.257 \pm 21.444$) which had an increased latency.

REARING

A dose effect was present, $F(2,58)= 40.682$, $p< .0001$. There was lowered rearing with the high dose ($5.229 \pm .78$) compared with saline ($16.171 \pm 1.216$)and the low dose ($13.286 \pm 1.093$).

STILL

There was a dose effect, $F(2,58)= 25.251$, $p<.0001$. Increased time was spent still at the high dose ($17.486 \pm 1.82$) compared with the saline ($4.943 \pm 1.183$) and the low dose ($7.143 \pm 1.279$).

WALKING

A sex difference was found, $F(1,29)= 20.390$, $p< .0001$. Females were

found to walk more than males (males= 12.686 ± .574, females= 16.259 ± .599).

A dose effect was also found, F(2,58)=12.625, p< .0001, however these sex and dose effects interacted, F(2,58)= 4.148, p< .05. This was due to a dose effect for females only, F(2,58)= 14.942, p< .0001. There was decreased walking with the high dose of diazepam compared with saline and the low dose of diazepam (saline = 18.889 ± 1.551, low dose = 19.389 ± 1.669 and the high dose = 10.5 ± .72).

## CAFFEINE

### AMBULATION

A sex effect was found, F(1,29)= 9.598, p< .005. Females had higher ambulation(49.481 ± .785 ) than males(44.118 ± 1.628).

There was a dose effect, F(2,58)= 31.383, p< .0001. Higher ambulation occurred with the low and high dose of caffeine compared with saline (saline = 39.8 ± 1.933, low dose = 50.029 ± .734 and high dose = 50.8 ± .905).

### CENTRE

A dose effect was found, F(2,58)= 27.590, p< .0001. Lower centre occupancy was found with the saline dose (5.437 ± .737) than with the low (10.429 ± .686) or high dose of caffeine (10.743 ± .496).

### CORNER

There was a dose effect, F(2,58)= 18.867, p< .0001, which showed that with the saline dose, higher corner occupancy occurred than with the low or

high dose of caffeine, (saline = 29.457 ± 1.556, low dose = 21.286 ± 1.055 and high dose = 21.171 ± .658).

DEFECATION

There was a sex effect, $F(1,29)= 23.024$, $p< .0001$, males defecated more than females (males = 4 ± .53, females = .944 ± 0.304).

EMERGENCE

A sex effect was present, $F(1,29)= 8.126$, $p< .01$. Males took longer to emerge than females, (males = 160.853 ± 18.121 and females = 91.352 ± 15.051).

GROOMING

A perinatal caffeine effect occurred, $F(2,29)= 4.458$, $p< .05$. The high perinatal caffeine exposed group groomed more than either the controls or the low perinatal caffeine group (high group = 2.806 ± .354, control = 1.606 ± .254, low group = 1.722 ± .309).

A sex x acute dose interaction existed, $F(2,58)= 3.434$, $p<.05$. Further analyses showed that this was partly due to a dose effect for males only, $F(2,58)= 4.247$, $p< .05$. There was lower grooming with the high dose of caffeine (1.294 ± .371) compared with saline (3.176 ± .787). However most of this interaction was due to a sex effect for saline treated animals only, $F(1,86)= 4.236$, $p< .05$, (males = 3.176 ± .787 and females = 1.778 ± .286).

REARING

There was a marginally significant perinatal caffeine effect, $F(2,29)= 3.230$, $p<.06$. There was higher rearing by the controls compared with the

high perinatal caffeine group and the low perinatal caffeine group (control = 18.515 ± 1.31, low group = 14.444 ± 1.09 and the high group mean = 14.917 ± 1.169).

STILL

A dose effect, $F(2,58)= 3.191$, $p< .05$, was found to be due to animals treated with the saline spending more time still than when given the low or high dose of caffeine (saline = 4.943 ± 1.183, low dose = 2.543 ± .547 and high dose = 2.343 ± .42).

WALKING

A sex effect was found, $F(1,29)= 15.044$, $p< .001$. Females had higher walking than the males (females = 22.815 ± .492, males = 19.157 ± 1.137)

There were two sets of interactions, sex x dose, $F(2,58)= 3.199$, $p<.05$, and sex x caffeine, $F(2.29)= 12.905$, $p< .0001$.

The sex x dose interaction was due to a sex effect at the saline dose, $F(1,82)= 4.596$, $p< .05$, and the high caffeine dose, $F(1,82)= 12.695$, $p< .001$, the females walked more than the males at the saline dose. At the high dose, the males walked more than the females (saline = 14.824 ± 1.29 for males and 18.889 ± 1.551 for females, at high dose = 22.059 ± 1.648 for males and 10.5 ± .72 for females).

There was a dose effect for males, $F(2,58)=7.561$, $p< .001$. Lower walking occurred with saline (14.824 ± 1.29) than with the low (22.059 ± 1.648) or high dose (20.588 ± 1.957).

A dose effect for females also existed, $F(2,58)= 10.187$, $p< .0001$. The high caffeine dose (27.722 ± 1.468) resulted in higher walking than saline (18.889 ± 1.551).

The sex x perinatal caffeine interaction was due to a perinatal caffeine effect for males but not females, $F(2,29)= 17.053$, $p< .0001$. The control group

had higher walking than the low perinatal caffeine group, and the high perinatal caffeine group. The low perinatal caffeine group also had higher walking than the high perinatal caffeine group (control = 23.933 ± 1.762, low group = 19.389 ± .611 and the high group mean was 14.944 ± 1.381).

There was also a sex effect for the low perinatal caffeine group, $F(1,29)= 4.515$, $p< .05$, and for the high perinatal caffeine group, $F(1,29)= 36.019$, $p< .0001$. Females walked more than males for both (low group = 22.556 ± 1.17 for females and 19.389 ± .611 for the males, high group = 23.889 ± .556 for females and 14.944 ± 1.381 for the males).

Figure 1(a). Effect of diazepam on ambulation by rats exposed perinatally to caffeine

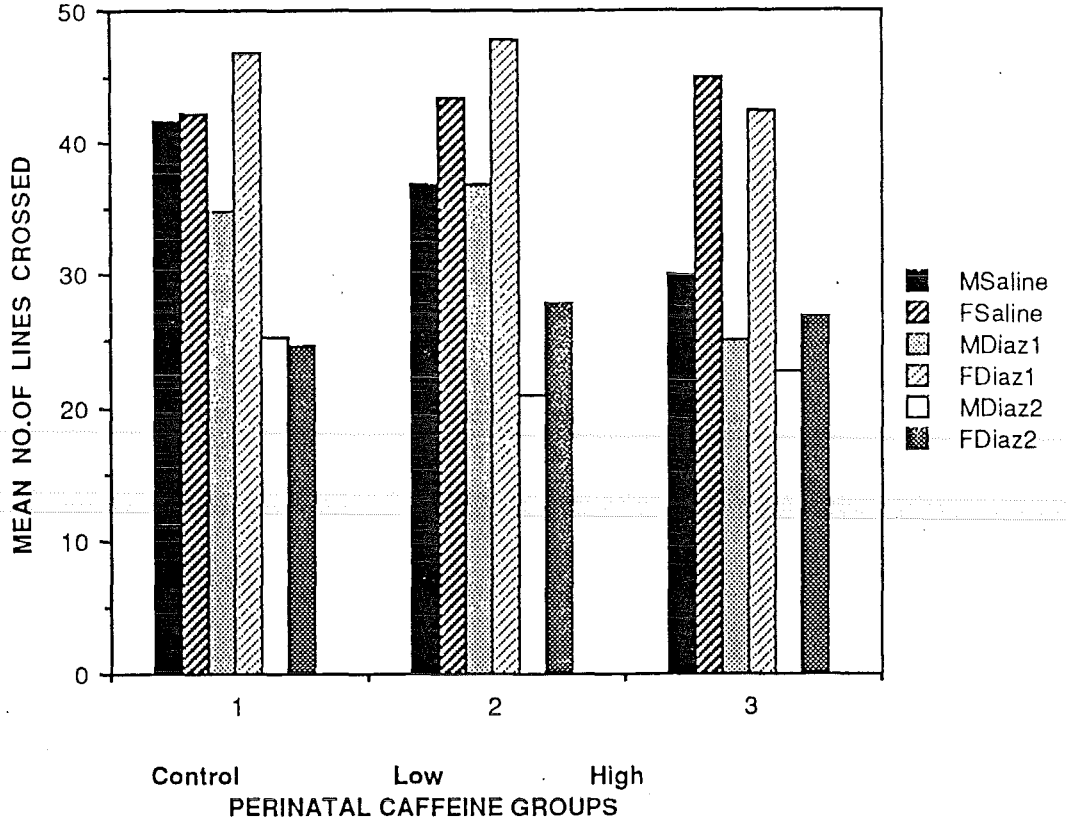



FIGURE 1(b). Effect of caffeine on ambulation by rats exposed perinatally to caffeine

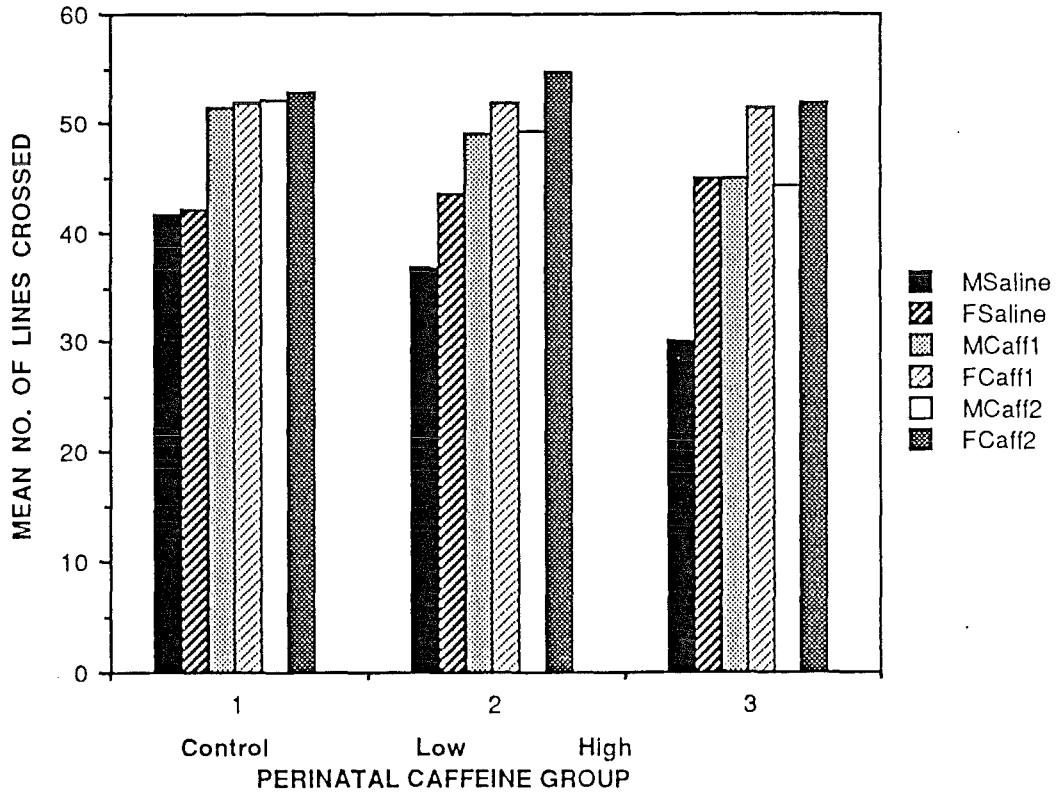


Figure 2(a). Effect of diazepam on centre occupancy by rats exposed perinatally to caffeine

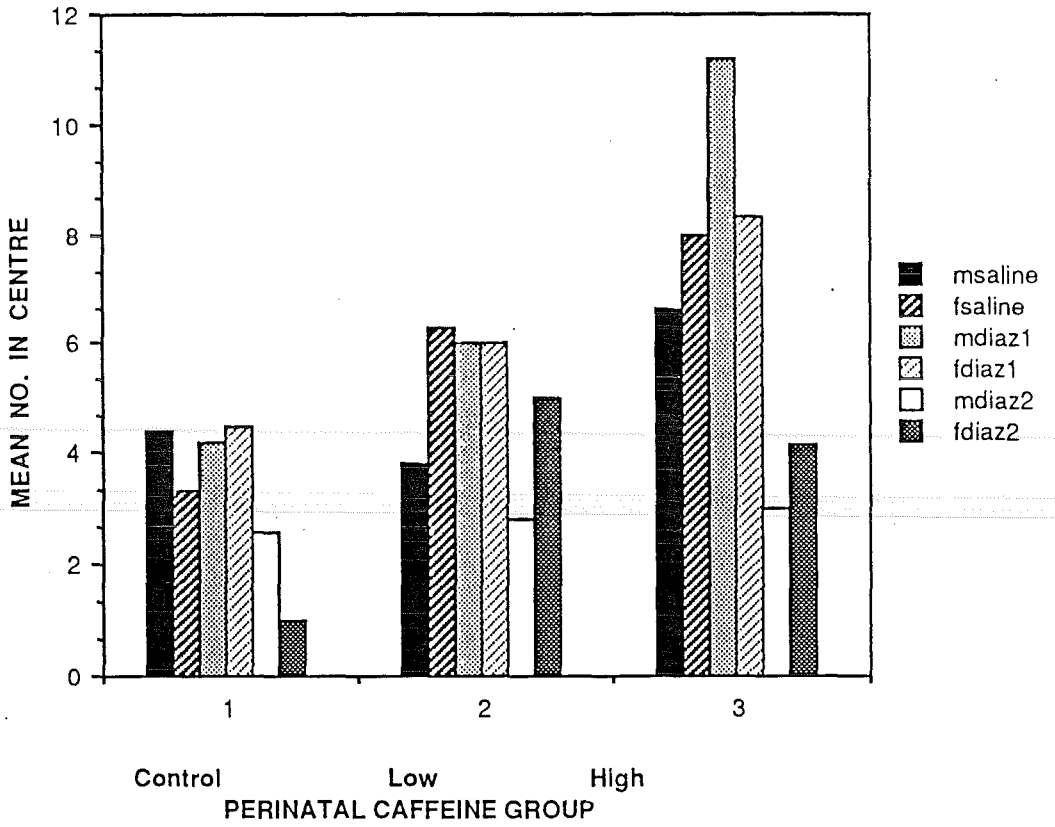



Figure 2(b). Effect of caffeine on centre occupancy by rats exposed perinatally to caffeine.

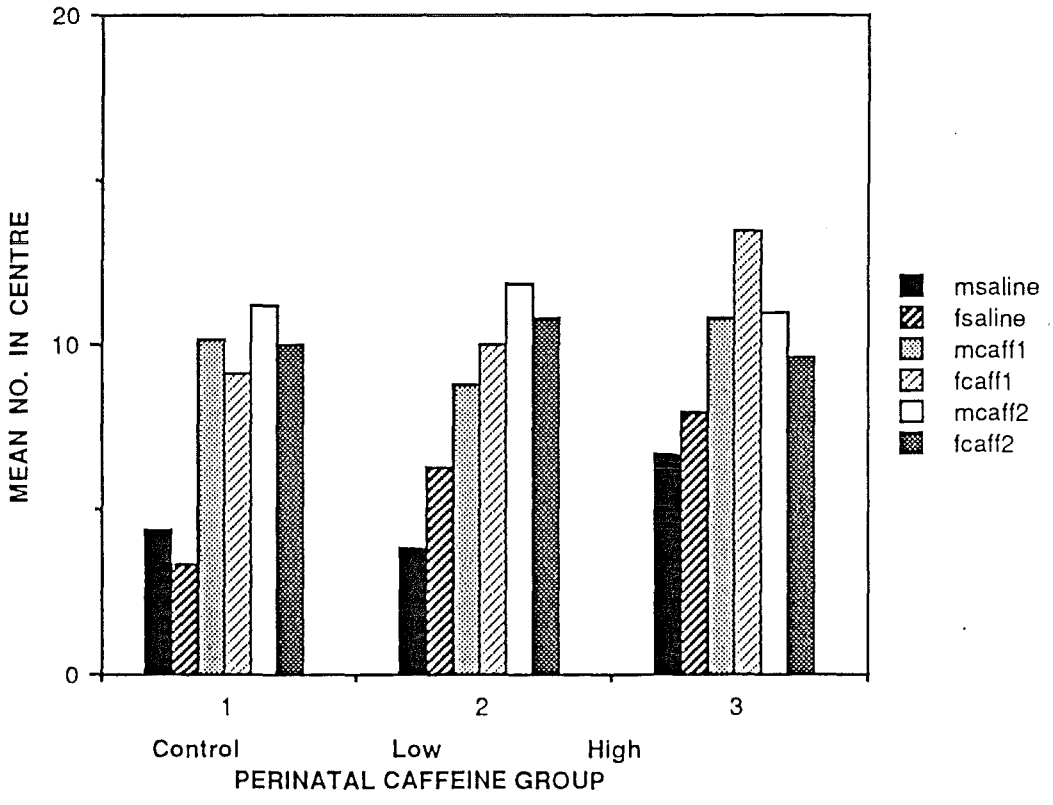


Figure 3(a). Effect of diazepam on corner occupancy by rats exposed perinatally to caffeine

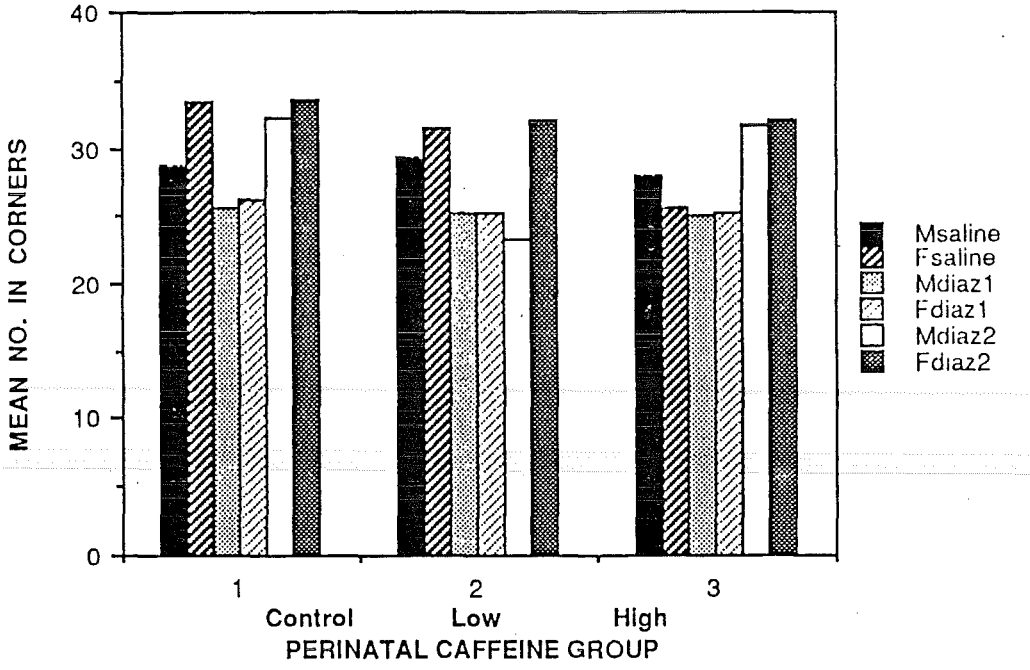



Figure 3(b). Effect of caffeine on corner occupancy by rats exposed perinatally to caffeine

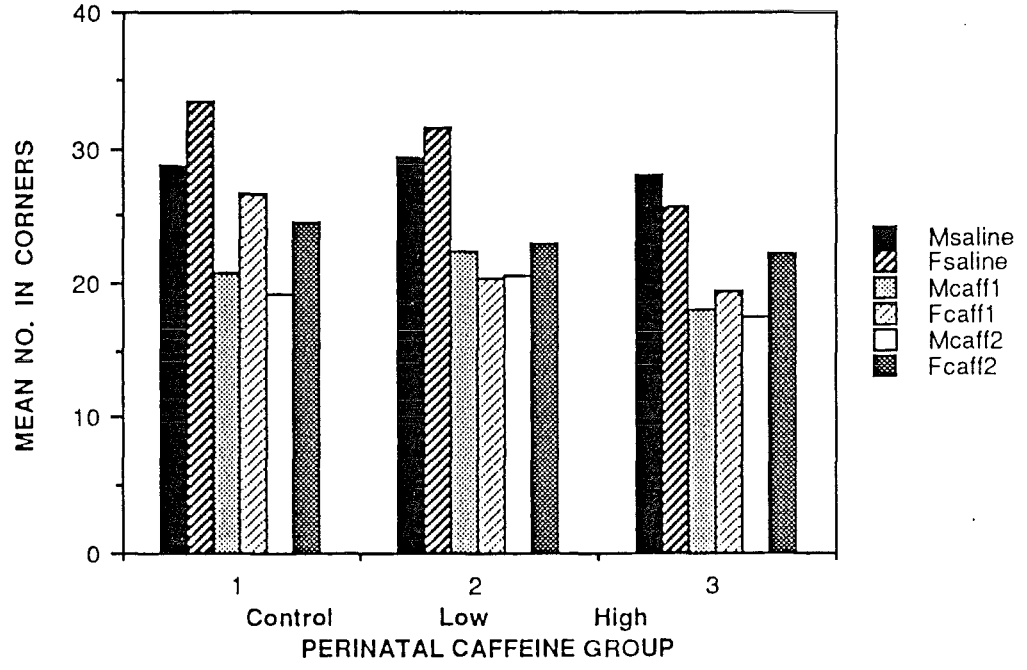


Figure 4(a). Effect of diazepam on defecation by rats exposed perinatally to caffeine

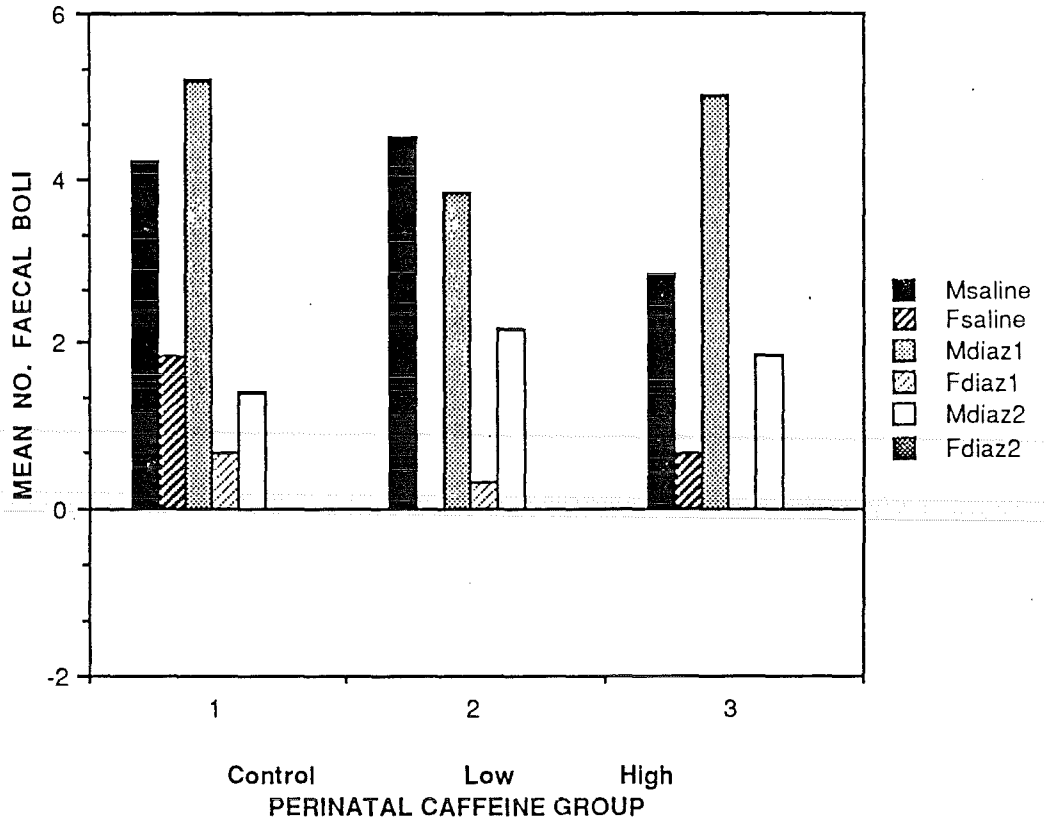



Figure 4(b). Effect of caffeine on defecation by rats perinatally exposed to caffeine

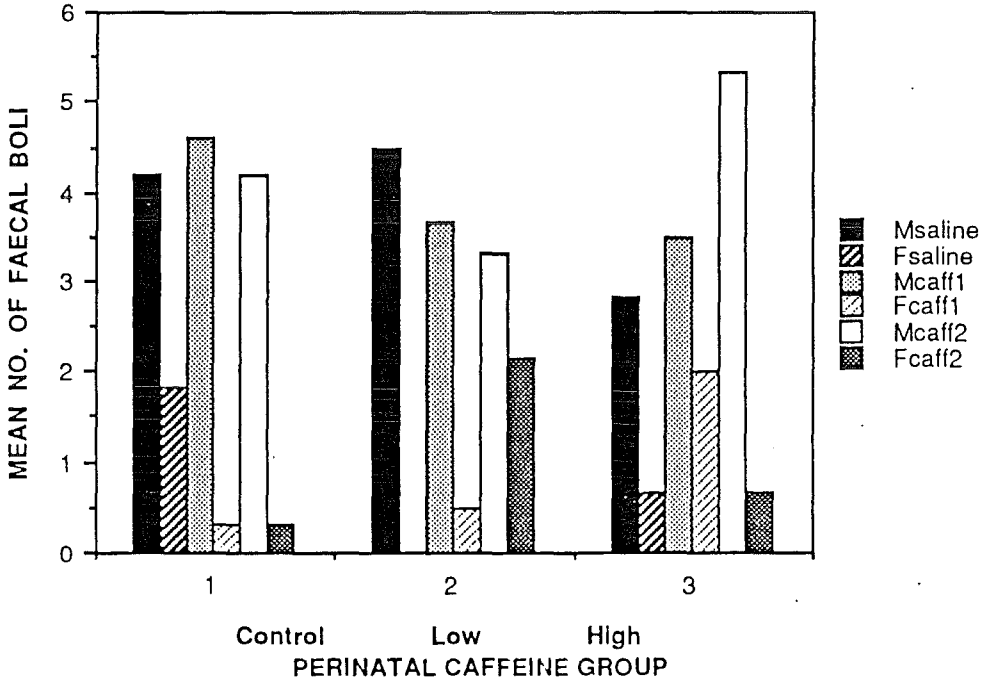


Figure 5(a). Effect of diazepam on emergence latency by rats perinatally exposed to caffeine

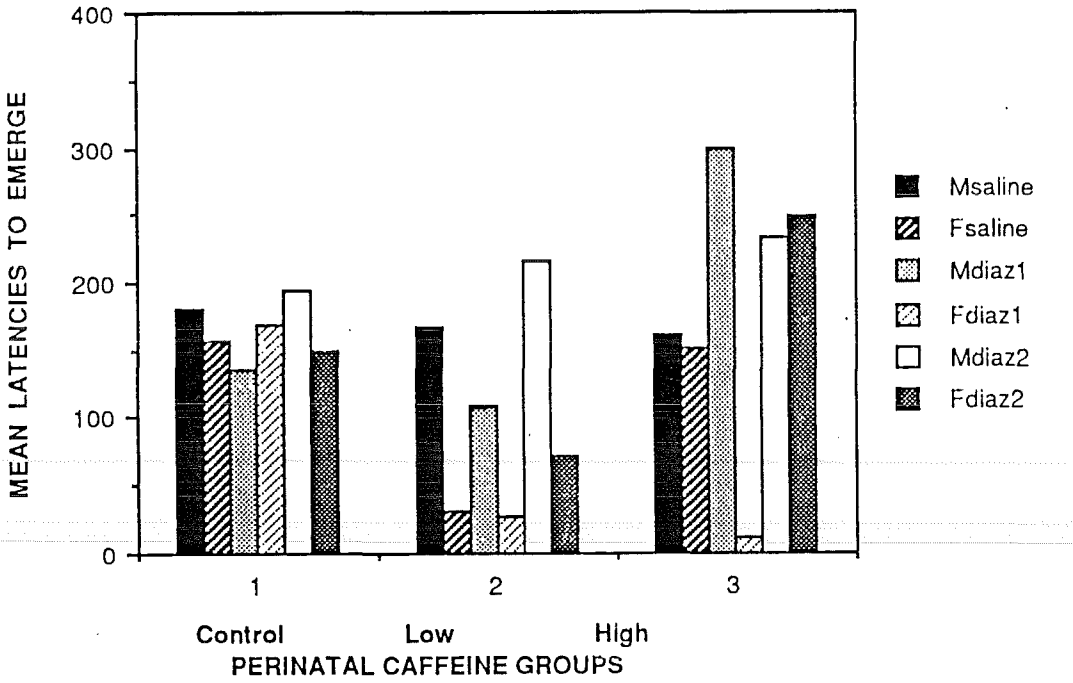



Figure 5(b). Effect of caffeine on emergence latencies by rats perinatally exposed to caffeine

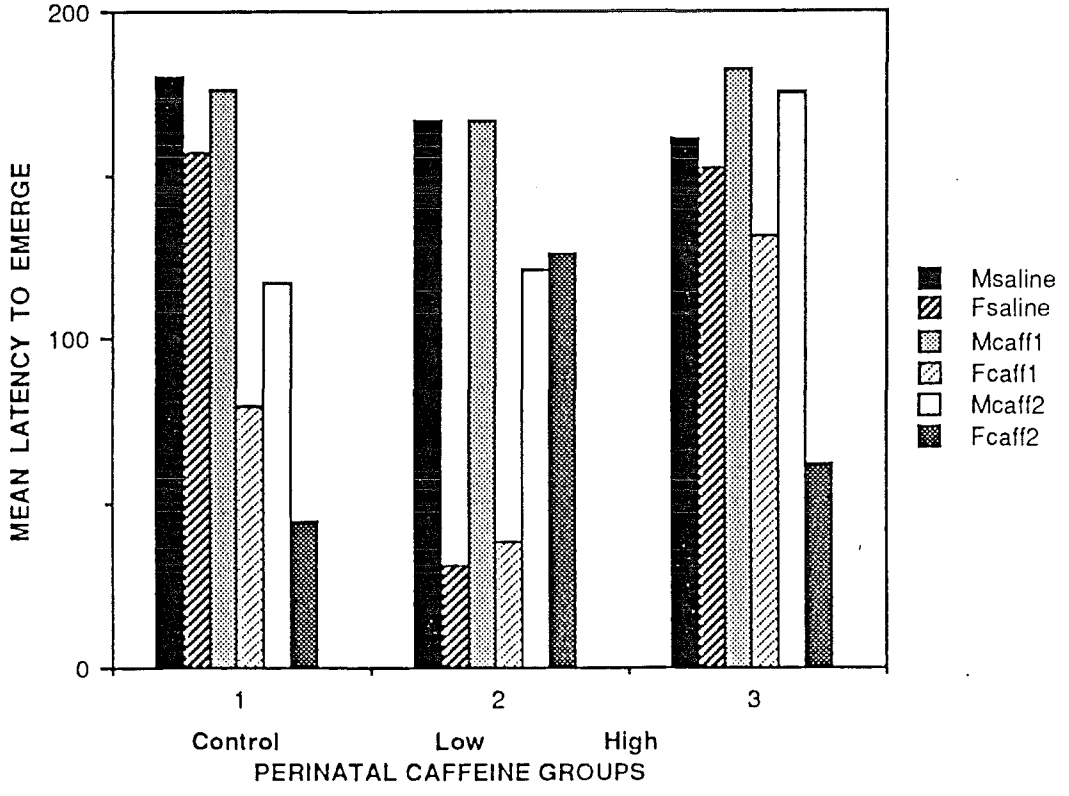


Figure 6(a). Effect of diazepam on grooming by rats perinatally exposed to caffeine

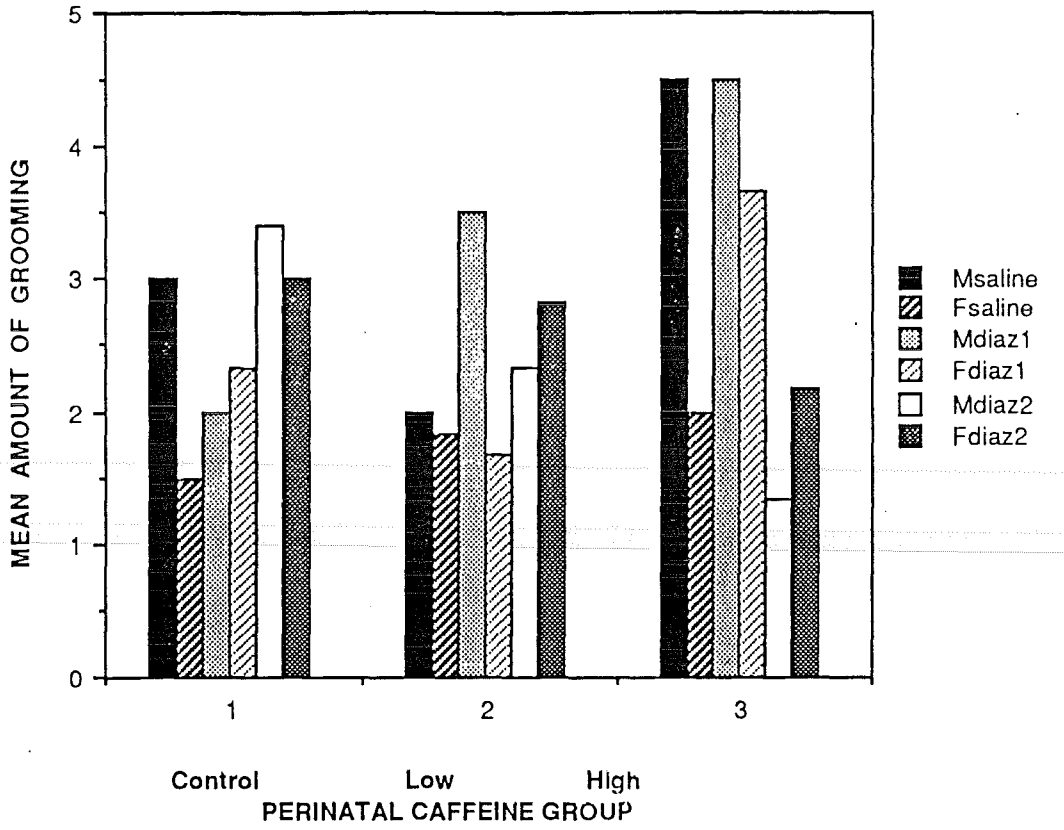



Figure 6(b). Effect of caffeine on grooming by rats perinatally exposed to caffeine

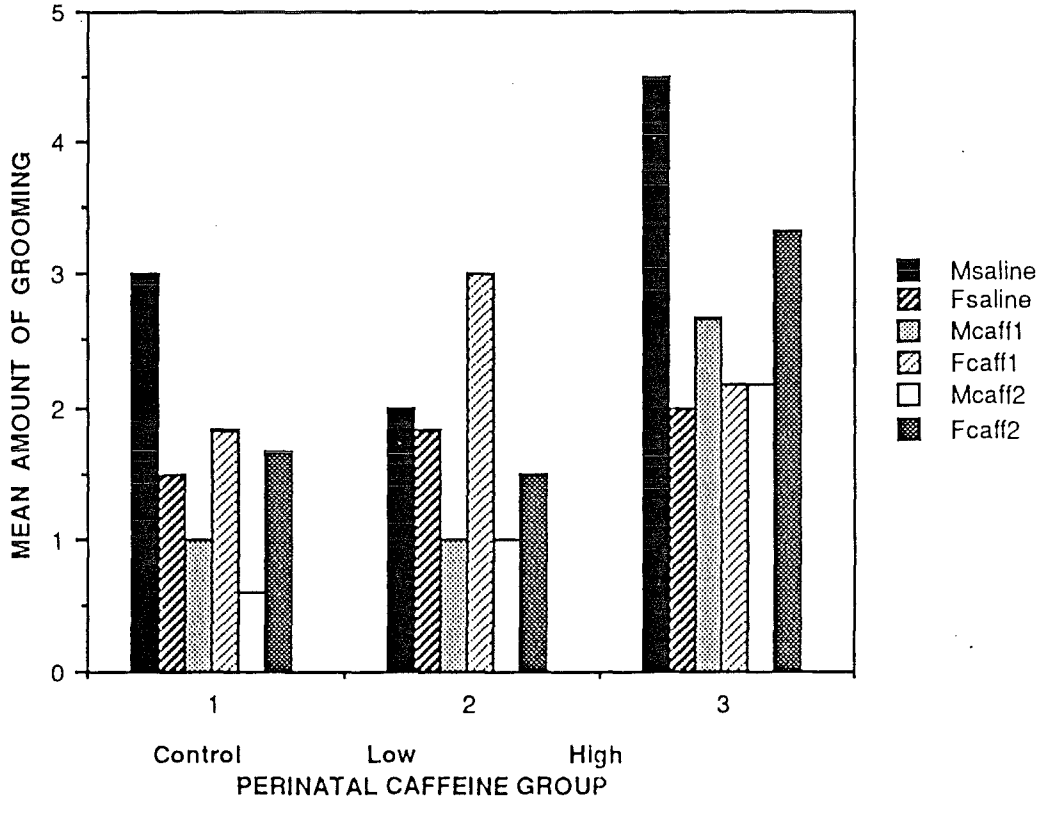


Figure 7(a). Effect of diazepam on rearing in rats perinatally exposed to caffeine

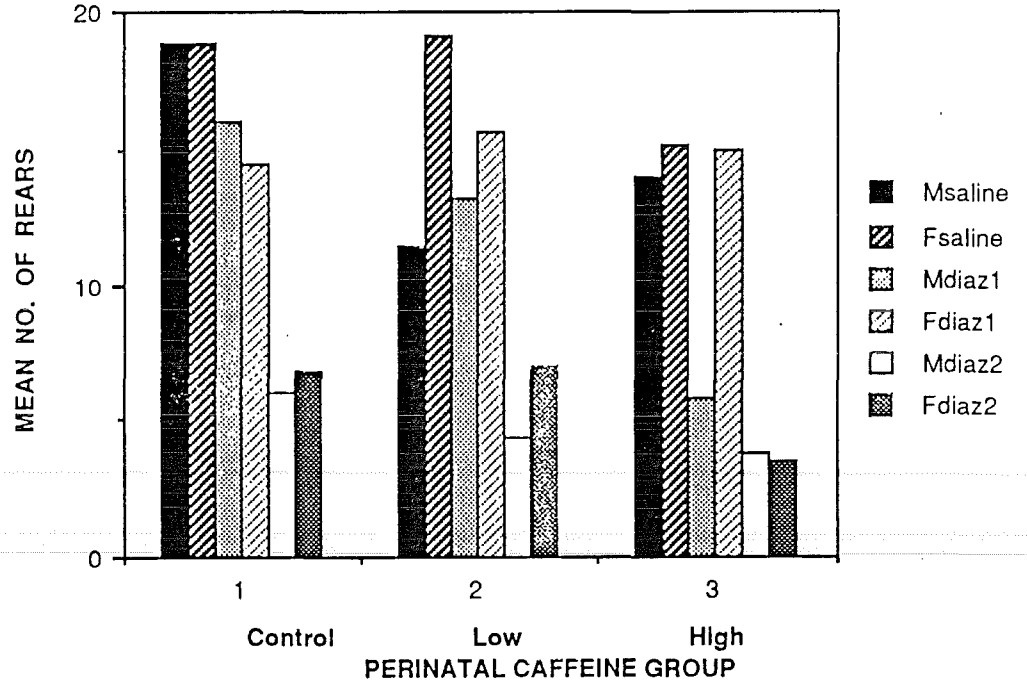



Figure 7(b). Effect of caffeine on rearing by rats exposed perinatally to caffeine

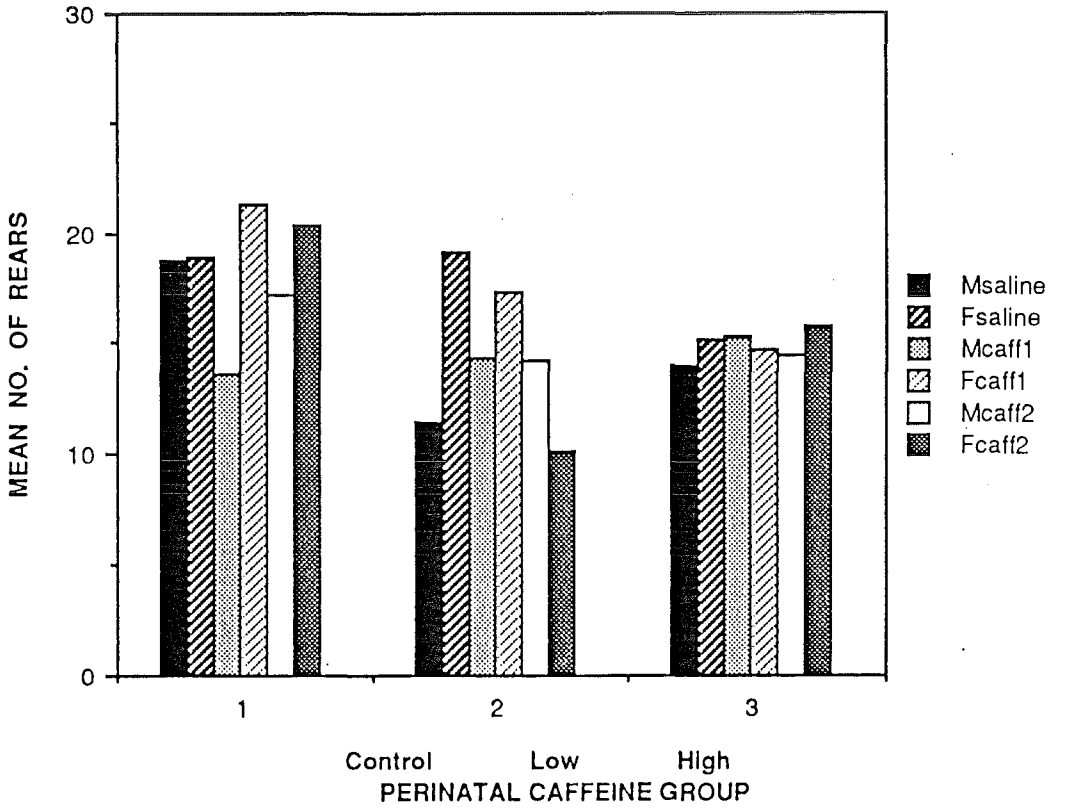


Figure 8(a). Effect of diazepam on still behaviour by rats exposed perinatally to caffeine

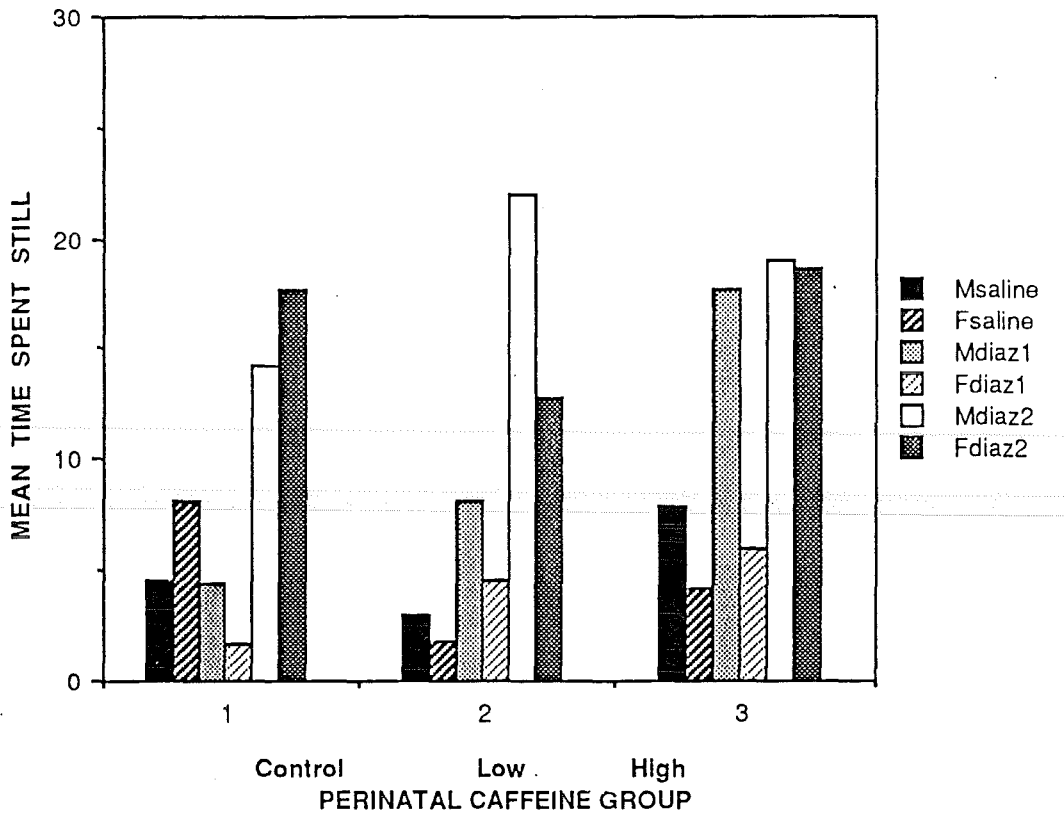



Figure 8(b). Effect of caffeine on still behaviour by rats exposed perinatally to caffeine

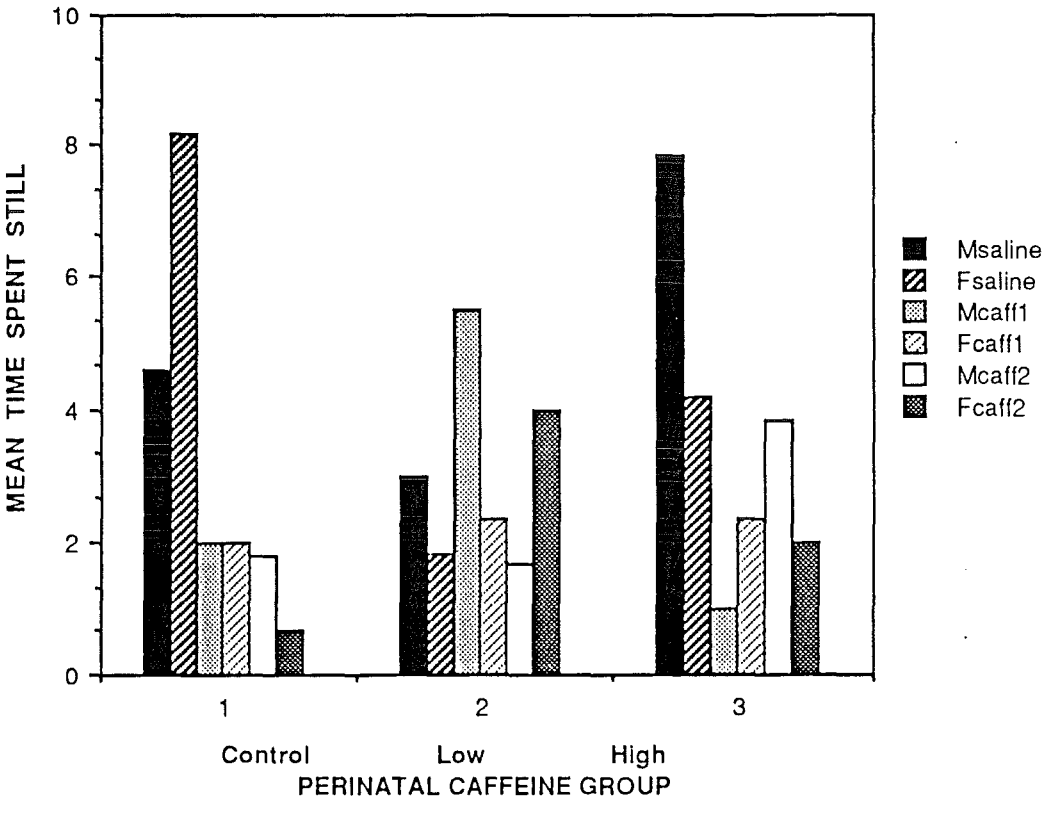


Figure 9(a). Effect of diazepam on walking by rats perinatally exposed to caffeine

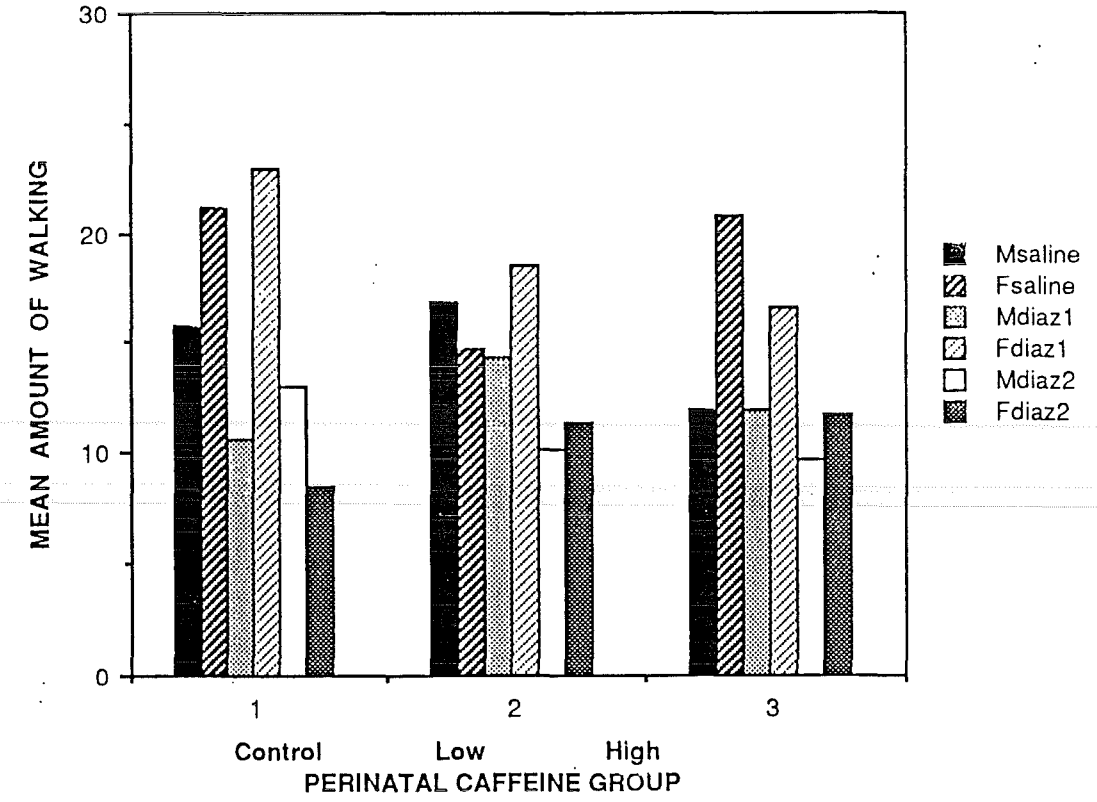
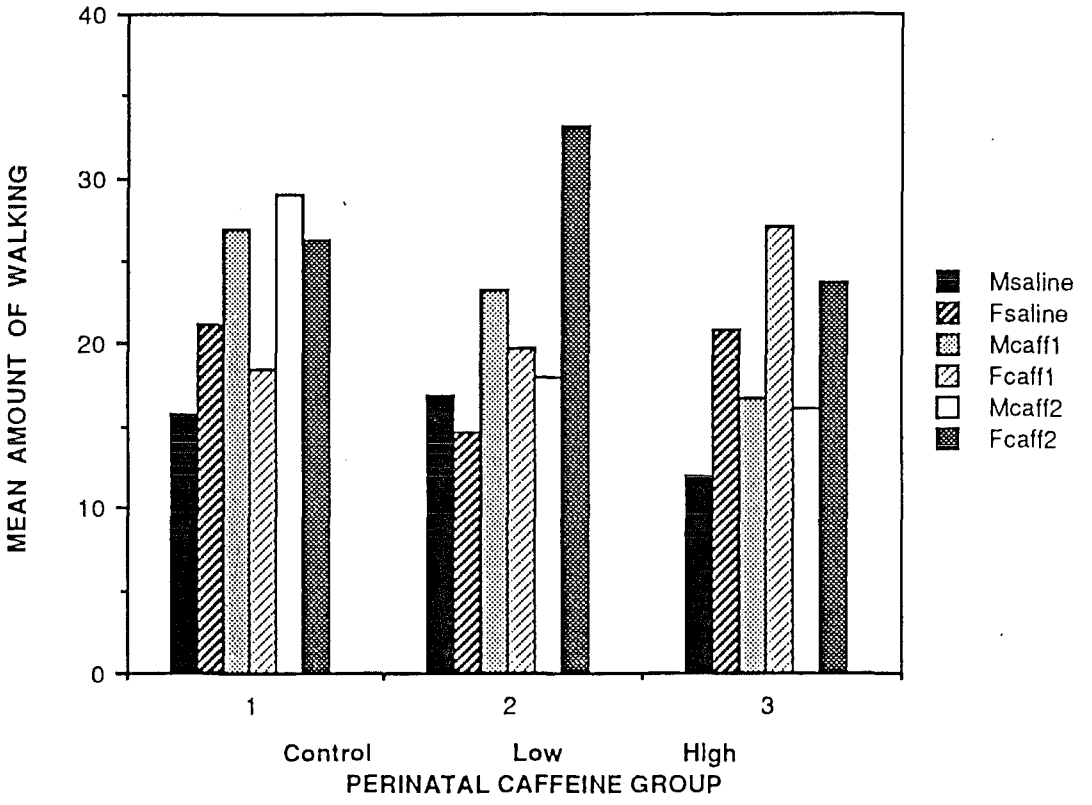



Figure 9(b). Effect of caffeine on walking by rats perinatally exposed to caffeine



# EXPERIMENT TWO

Effects on behavioural measures, with administration of saline, CHA and oxprenolol at low and high doses after exposure to perinatal caffeine,for each sex are outlined in Figures 10 - 18. These follow after the written results.

## CHLOROHEXYLADENOSINE (CHA)

### AMBULATION

Sex effects were found, $F(1,29)= 13.062$, $p< .002$. Females had higher ambulation scores than males (36.608 ± 1.196 for females, 21.093 ± 1.419 for males.

A dose effect occurred, $F(2,58)= 36.630$, $p< .0001$. There was higher ambulation with the saline dose (39.086 ± 1.707) than the low (27.543 ± 2.261) or high dose (19.257 ± 2.047), and there was also higher ambulation with the low dose than with the high dose of chlorohexyladenosine.

### CENTRE OCCUPANCY

A dose x perinatal caffeine interaction occurred, $F(4,58)= 3.061$,$p< .05$. This was accounted for by a dose effect for the high perinatal caffeine group, $F(2,58)= 6.178$, $p< .005$. There was more centre occupancy by the high caffeine group at the high dose than at the low dose, 11.545 ± 3.399(high dose) and 4.091 ± .857(low dose).

### DEFECATION

An overall sex effect, $F(1,29)= 17.556$, $p< .0005$, showed that males

have higher defecation than females (2.648 ± .283 for males and 1 ± .241 for females).

A further sex x dose interaction revealed that there was a dose effect for males only, $F(2,58)= 17.223$, $p< .0001$. Decreased defecation was found with both the low and high dose of CHA, (1.667 ± .548 for the low dose and 1.333 ± .404 for the high dose).

EMERGENCE

Males took longer to emerge than females, $F(1,29)= 14.156$, $p< .001$, (289.519 ± 7.193 for males, 193.078 ± 26.511 for females).

GROOMING

A sex effect, $F(1,29)= 3.926$, $p< .06$, revealed that males were found to groom more than females overall, (males = 3.889 ± .778, females = 2.137 ± .308).

There was a perinatal caffeine x dose interaction found, $F(4,58)= 3.811$, $p< .01$. This occurred because of a dose effect for the high perinatal caffeine group, $F(2,58)= 3.897$, $p< .05$. There was lower grooming by this group with the high dose of CHA (saline = 4.727 ± 1.01, low dose = 2.364 ± 1.089, high dose = 1.091 ± .476).

REARING

There was a sex effect, $F(1,29)= 16.325$, $p< .0005$, females reared more than males (females = 9.078 ± .837, males = 5.148 ± .602).

A dose effect, $F(2,58)= 43.039$, $p< .0001$, revealed that more rearing occurred with the saline dose than with either the low or high dose. The low dose gave greater rearing than the high dose (saline = 11.6 ± .908, low dose = 6.686 ±.892, high dose = 2.886 ± .584).

STILL

A sex effect was found, $F(1,29) = 31.236$, $p < .0001$, males spent more time still than females (males = 18.167 ± 2.089, females = 5.333 ± .828).

A dose effect, $F(2,58) = 32.390$, $p < .0001$, revealed that more time was spent still with the low and high dose than with the saline and more time was spent still with the high dose than with the low dose, (saline = 3.4 ± .764, low dose = 11.914 ± 2.2, high dose = 20.486 ± 2.75).

A sex effect at low and high dose showed that males spent more time still at the low dose, $F(1,80) = 19.188$, $p < .0001$ and at the high dose, $F(1,80) = 39.579$, $p < .0001$. Means for the low dose = 19.056 ± 3.444 for males, 4.353 ± .939 for females, high dose = 30.611 ± 3.667 for males and 9.765 ± 1.975 for females.

WALKING

Females showed more walking than males, $F(1,29) = 96.803$, $p < .0001$, (females = 14.745 ± .495, males = 7.981 ± .497).

A dose effect, $F(2,58) = 18.788$, $p < .0001$, showed that there was more walking with the saline than with the low or high dose of CHA (saline = 14.771 ± .947, low dose = 10.886 ± 1.009, high dose = 8.143 ± .807).

## OXPRENOLOL

AMBULATION

A sex effect was found, $F(1,28) = 36.425$, $p < .0001$. Females had higher ambulation scores than males, (males = 34.185 ± 1.444, females = 44.49 ± .967.

There was a dose effect, $F(2,56) = 4.160$, $p < .05$. The low dose resulted in higher ambulation than the high dose, (low dose = 41.853 ±1.239, high dose = 36.294 ± 1.767.

A sex x perinatal caffeine interaction occurred, $F(2,28) = 2.949$, $p < .07$. This was due to a sex effect for the controls, (males = 36.278 ± 2.114, females = 44.889 ± 2.167) , the low perinatal caffeine group (males = 36.333 ± 2.434, females = 42.833 ± 1.556) and the high perinatal caffeine group, (males = 29.944 ± 2.34, females = 46 ± .775). Males had lower ambulation in all three groups .

CENTRE

Females had higher centre occupancy than males, $F(1,28) = 3.738$, $p < .05$ (females = 6.039 ± .565, males = 4.815 ± .578.

There was a sex x perinatal caffeine interaction, $F(2,28) = 4.470$, $p < .05$. This was due to a caffeine effect for females only, $F(2,28) = 5.327$, $p < .05$. The high perinatal caffeine group spent more time in the centre than the controls or the low perinatal caffeine group. The mean for controls = 4.944 ± .617, low group = 5.056 ± .691, high group = 8.533 ± .923.

DEFECATION

There was a sex effect, $F(1,28) = 49.337$, $p < .0001$. Males were found to defecate more than females, (males = 3.204 ± .267, females = .863 ± .28.

A dose effect, $F(2,56) = 13.659$, $p < .0001$, showed that there was higher defecation with the saline dose than with the low or high dose of oxprenolol, (saline dose = 3.471 ± .486, low dose = 1.706 ± .399, high dose = .941 ± .242).

A sex x perinatal caffeine effect, $F(2,28) = 3.449$, $p < .05$, indicated that there was a caffeine effect for the males only, $F(2,28) = 3.410$, $p < .05$. The low perinatal caffeine group defecated less than the controls (low group = 2.556 ± .294, control group = 4.056 ± .467).

EMERGENCE

There was a sex effect, $F(1,28) = 22.719$, $p < .0001$. Males took longer to emerge than females, (males = 279.907 ± 10.848, females = 147.922 ± 27.336).

GROOMING

A sex effect, $F(1,28) = 19.174$, $p < .0005$, showed that males groomed more often than females, (males = 3.833 ± .415, females = 1.725 ± .404).

REARING

There was a sex effect, $F(1,28) = 16.642$, $p < .0005$. Females reared more than males, (females = 13.784 ± .908, males = 9.981 ± .685).

A marginal caffeine effect was found, $F(2,28) = 2.905$, $p < .075$. The low perinatal caffeine group had lower rearing than the control group. The mean for the low group = 10.694 ± .717 and the control group = 13.694 ± 1.1).

STILL

There was a sex effect, $F(1,28) = 6.978$, $p < .02$. Males spent more time still than females, (males = 3.13 ± .742, females = 1.02 ± .222).

A dose effect, $F(2,56) = 6.464$, $p < .005$, showed that more time is spent still with the saline dose than with the low dose (saline = 3.441 ± .786, low dose = .853 ± .261).

WALKING

Females walked more than males, $F(1,28) = 20.840$, $p < .0001$ (females = 17.784 ± .788, males = 12.833 ± .606).

EMERGENCE

There was a sex effect, $F(1,28) = 22.719$, $p < .0001$. Males took longer to emerge than females, (males = 279.907 ± 10.848, females = 147.922 ± 27.336).

GROOMING

A sex effect, $F(1,28) = 19.174$, $p < .0005$, showed that males groomed more often than females, (males = 3.833 ± .415, females = 1.725 ± .404).

REARING

There was a sex effect, $F(1,28) = 16.642$, $p < .0005$. Females reared more than males, (females = 13.784 ± .908, males = 9.981 ± .685).

A marginal caffeine effect was found, $F(2,28) = 2.905$, $p < .075$. The low perinatal caffeine group had lower rearing than the control group. The mean for the low group = 10.694 ± .717 and the control group = 13.694 ± 1.1).

STILL

There was a sex effect, $F(1,28) = 6.978$, $p < .02$. Males spent more time still than females, (males = 3.13 ± .742, females = 1.02 ± .222).

A dose effect, $F(2,56) = 6.464$, $p < .005$, showed that more time is spent still with the saline dose than with the low dose (saline = 3.441 ± .786, low dose = .853 ± .261).

WALKING

Females walked more than males, $F(1,28) = 20.840$, $p < .0001$ (females = 17.784 ± .788, males = 12.833 ± .606).

Figure 10(a). Effect of CHA on ambulation by rats perinatally exposed to caffeine

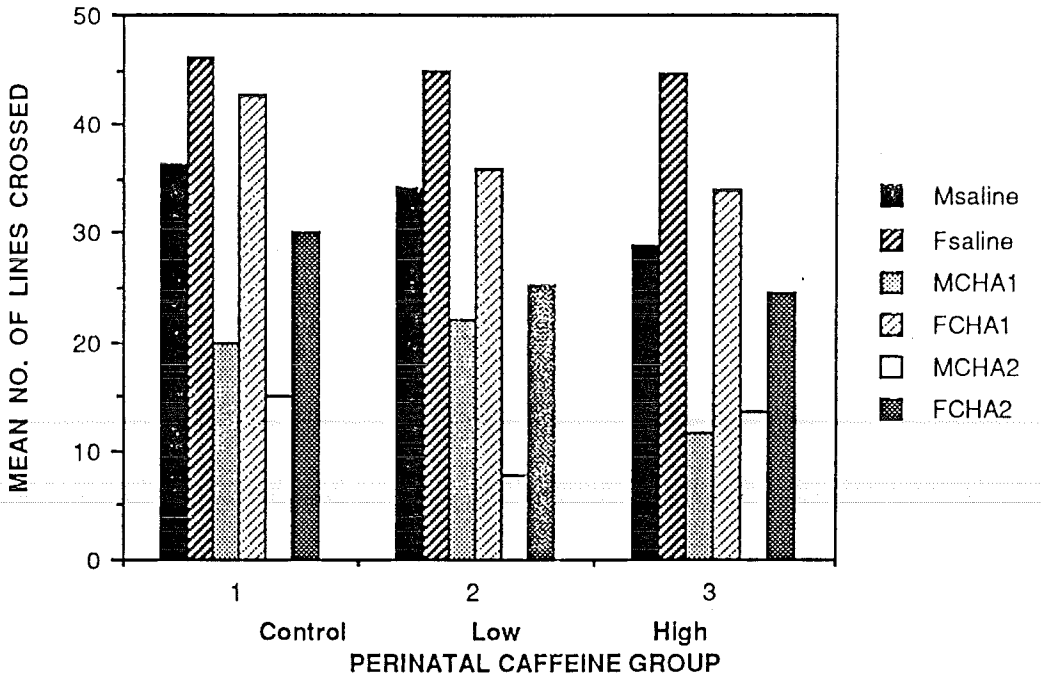



Figure 10(b). Effect of oxprenolol on ambulation by rats perinatally exposed to caffeine

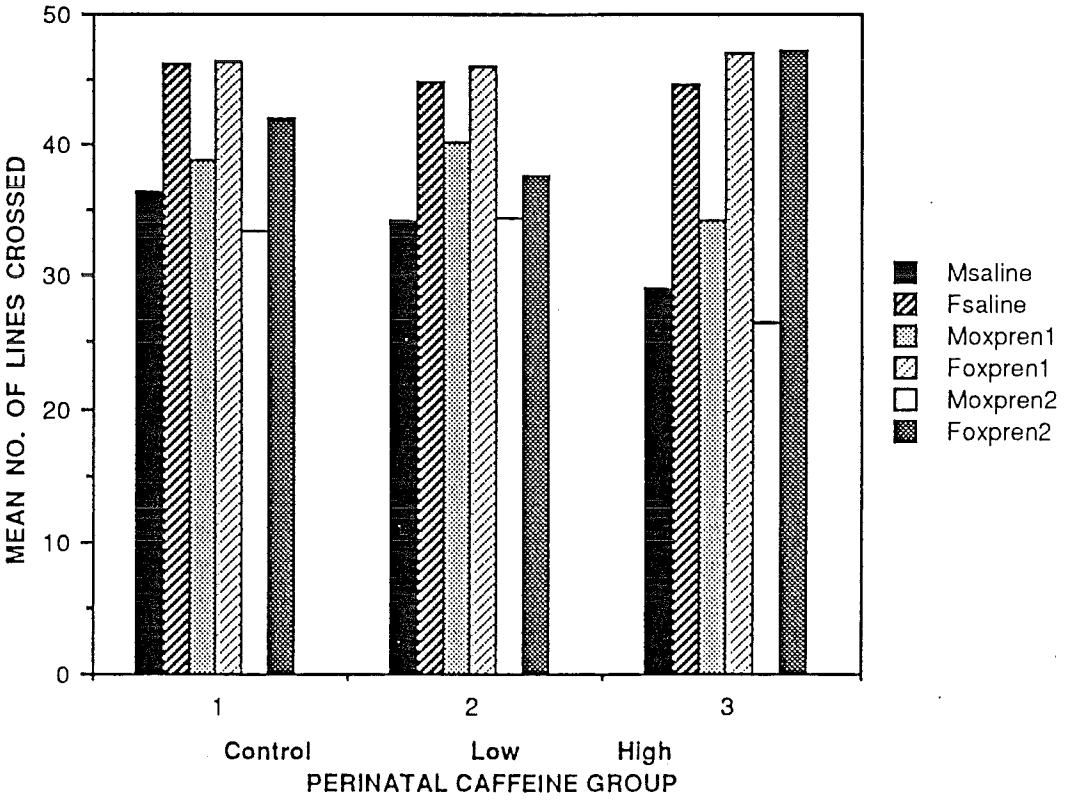


Figure 11(a). Effect of CHA on centre occupancy by rats exposed to perinatal caffeine

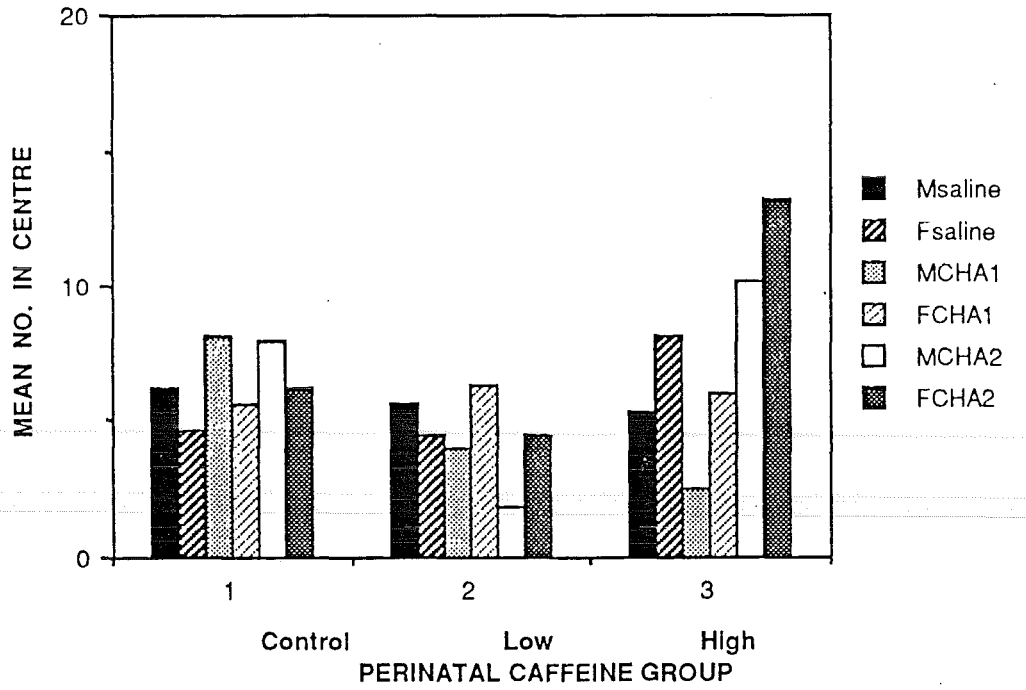



Figure 11(b). Effect of oxprenolol on centre occupancy in rats exposed perinatally to caffeine

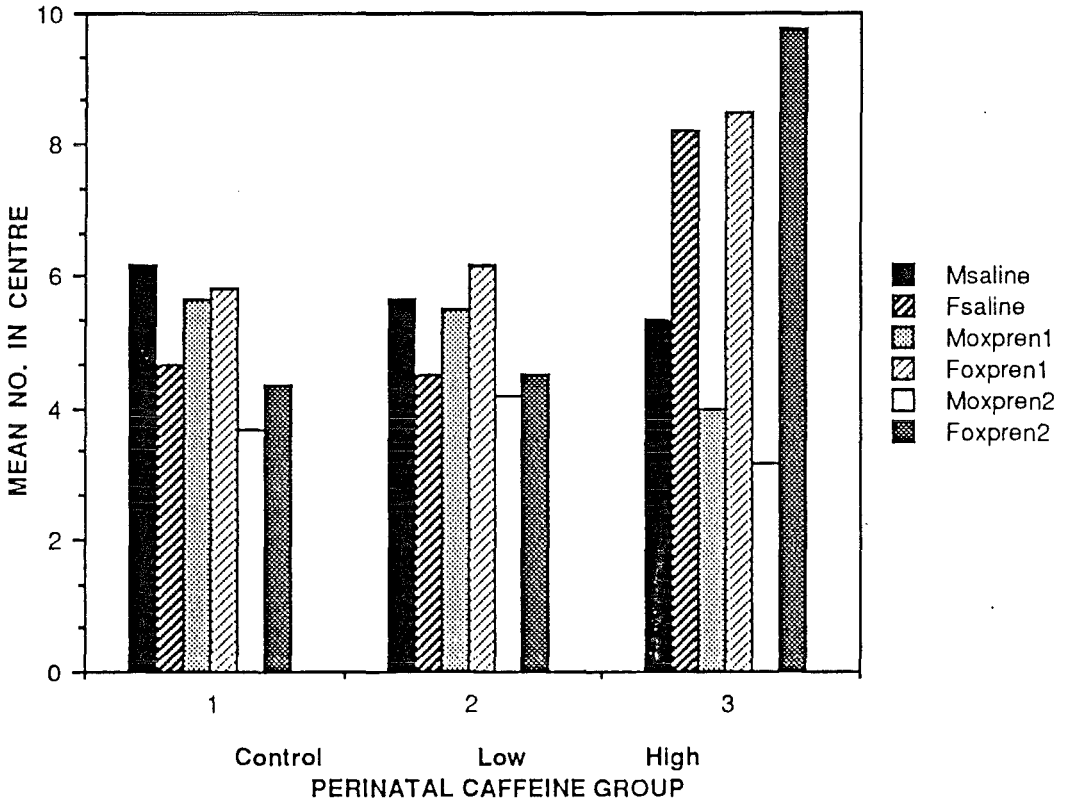


Figure 12(a). Effect of CHA on corner occupancy perinatally exposed to caffeine

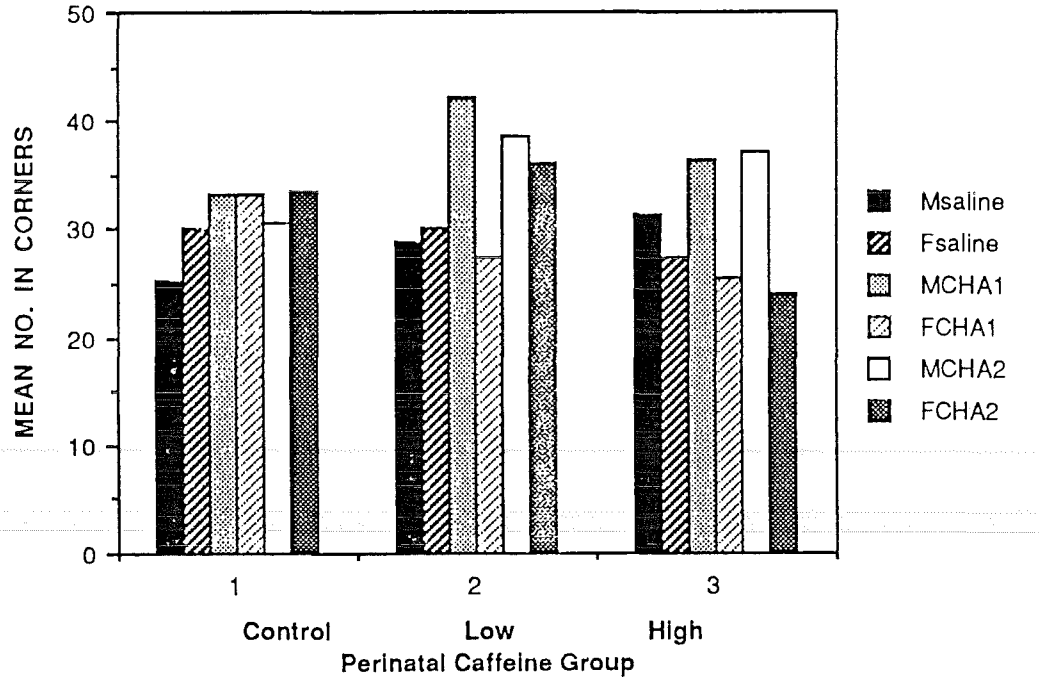



Figure 12(b). Effect of oxprenolol on corner occupancy by rats perinatally exposed to caffeine

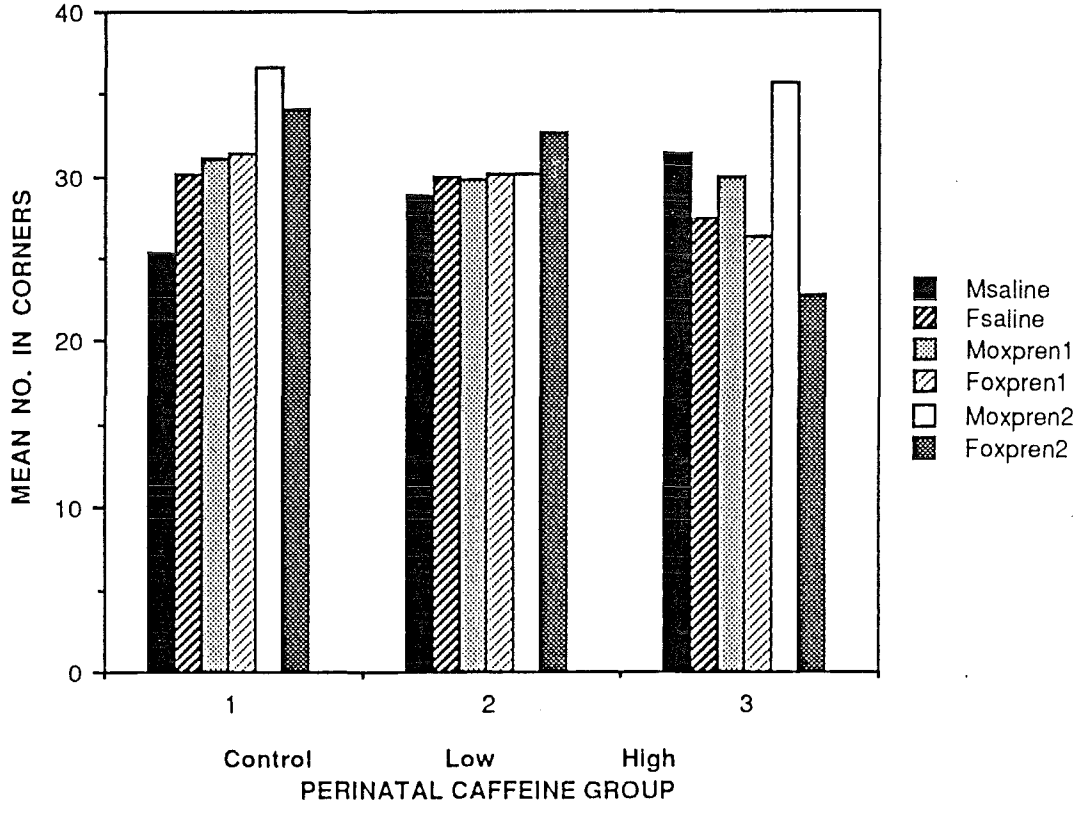


Figure 13(a). Effect of CHA on defecation by rats exposed perinatally to caffeine

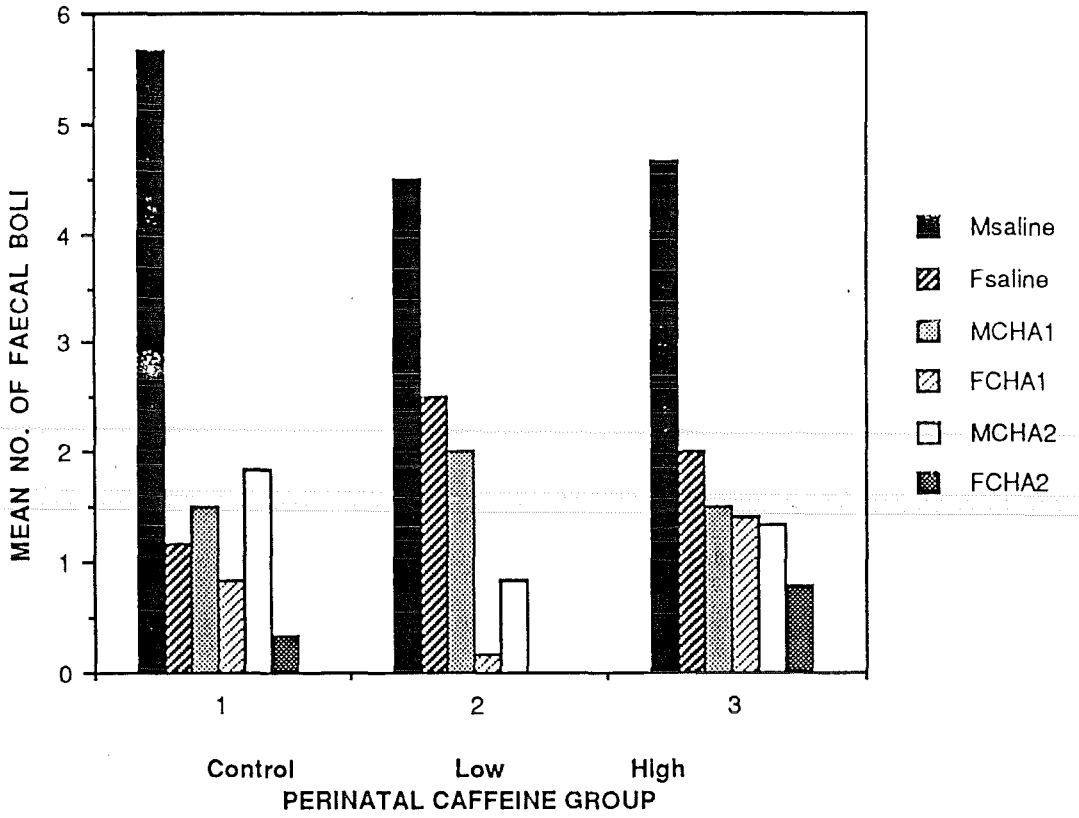



Figure 13(b). Effect of oxprenolol on defecation by rats exposed perinatally to caffeine

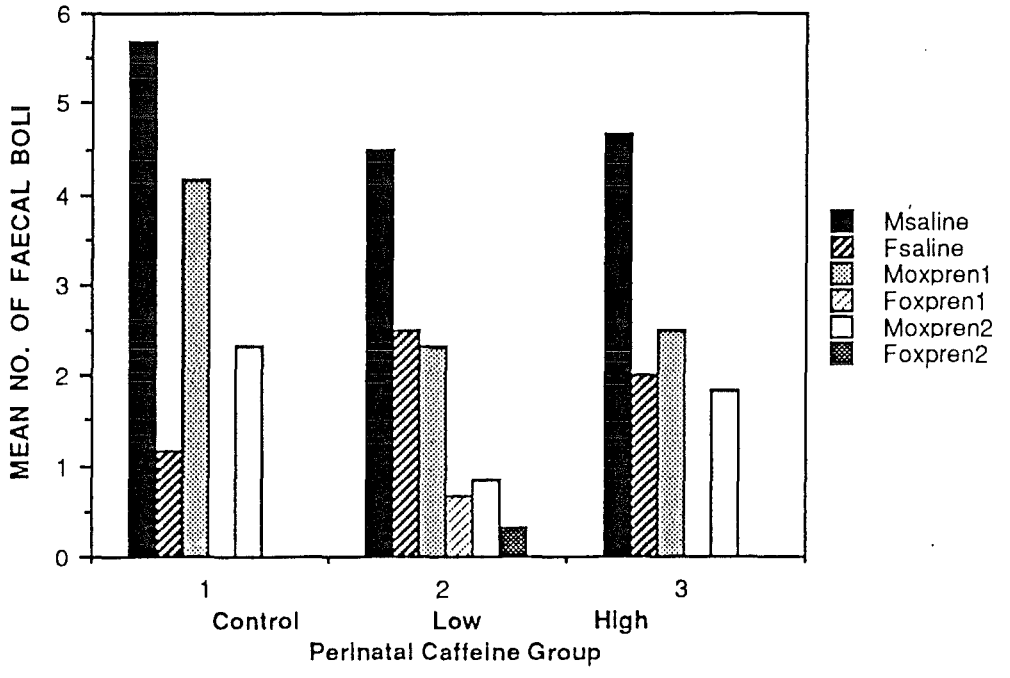


Figure 14(a). Effect of CHA on emergence latencies by rats exposed perinatally to caffeine

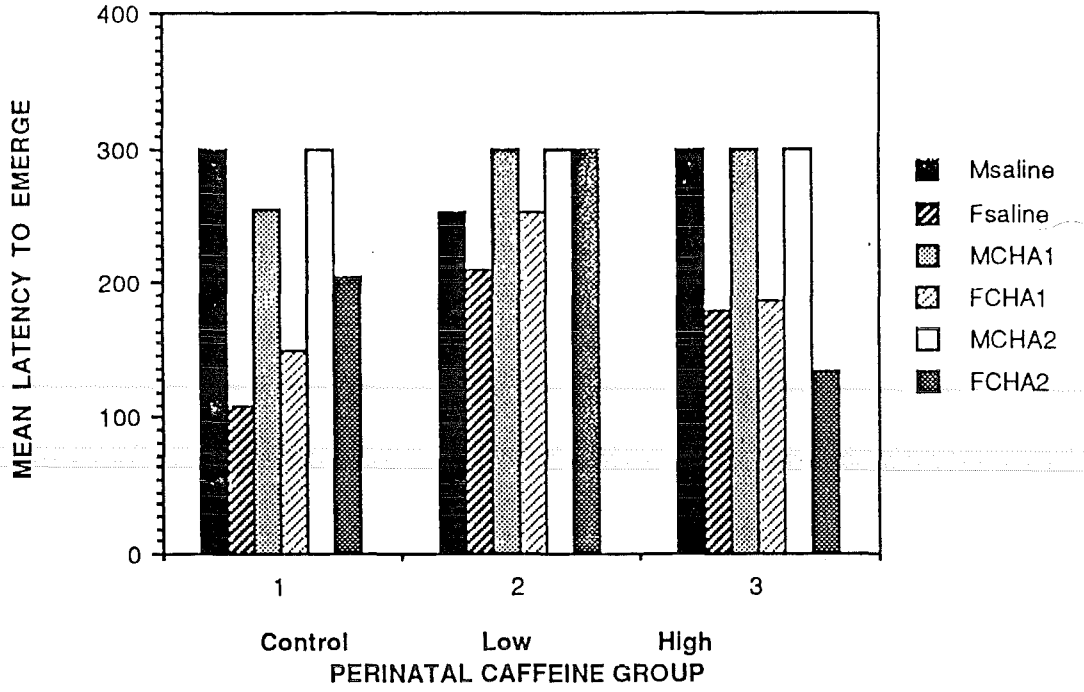



Figure 14(b). Effect of oxprenolol on emergence latencies by rats perinatally exposed to caffeine

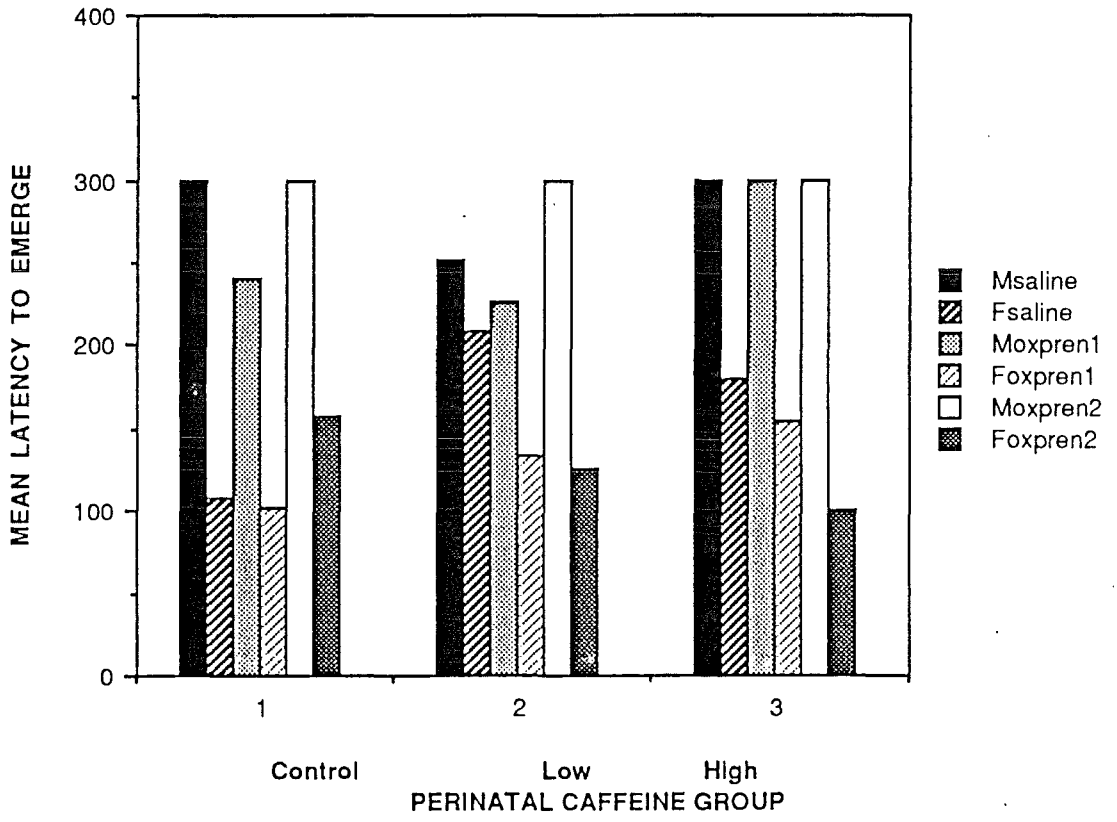


Figure 15(a). Effect of CHA on grooming in rats perinatally exposed to caffeine

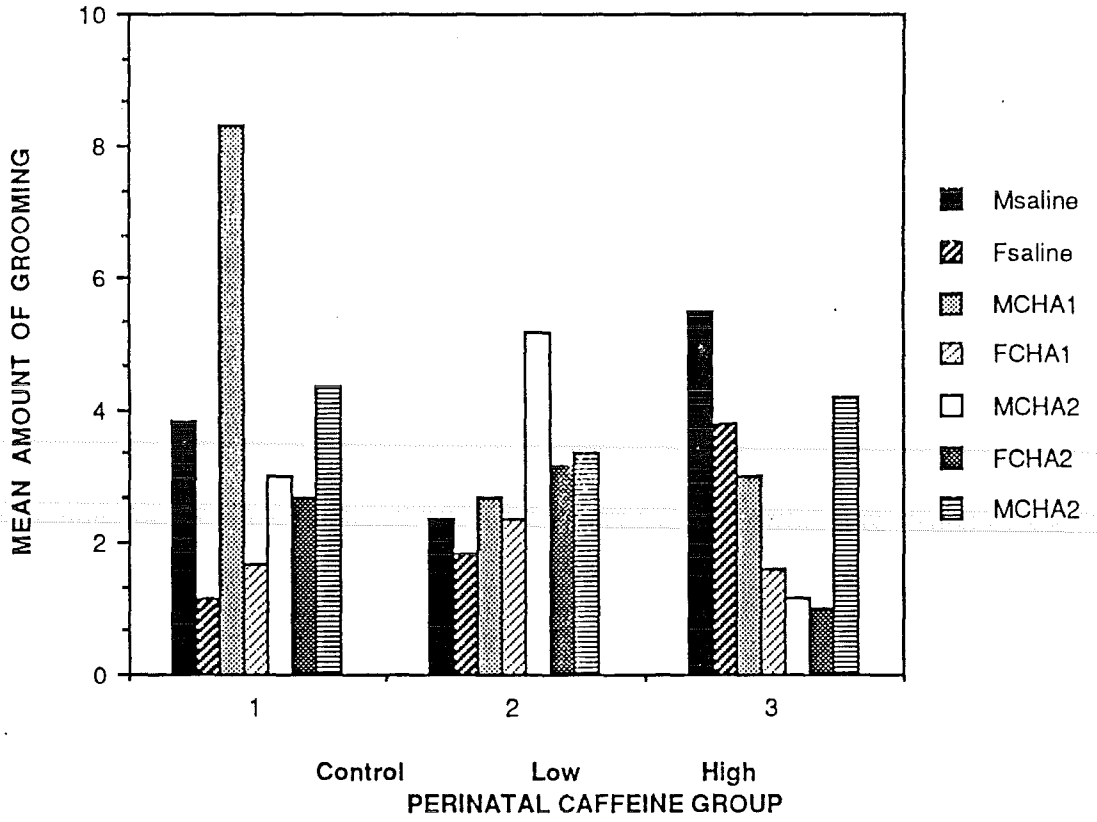



Figure 15(b). Effect of oxprenolol on grooming by rats exposed perinatally to caffeine

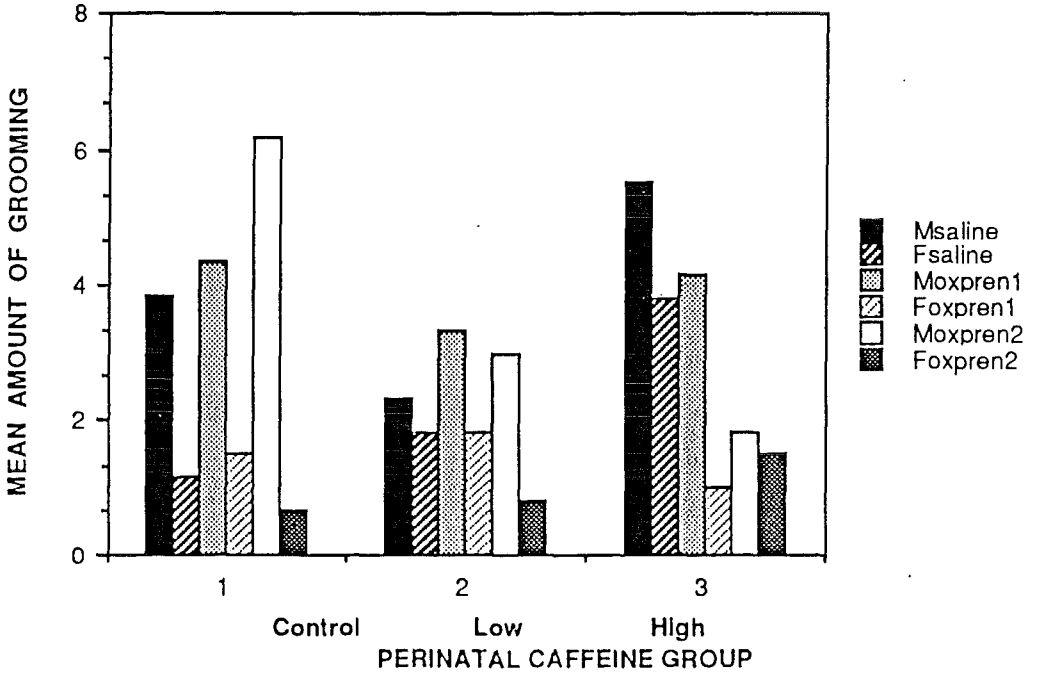


Figure 16(a). Effect of CHA on rearing by rats perinatally treated with caffeine

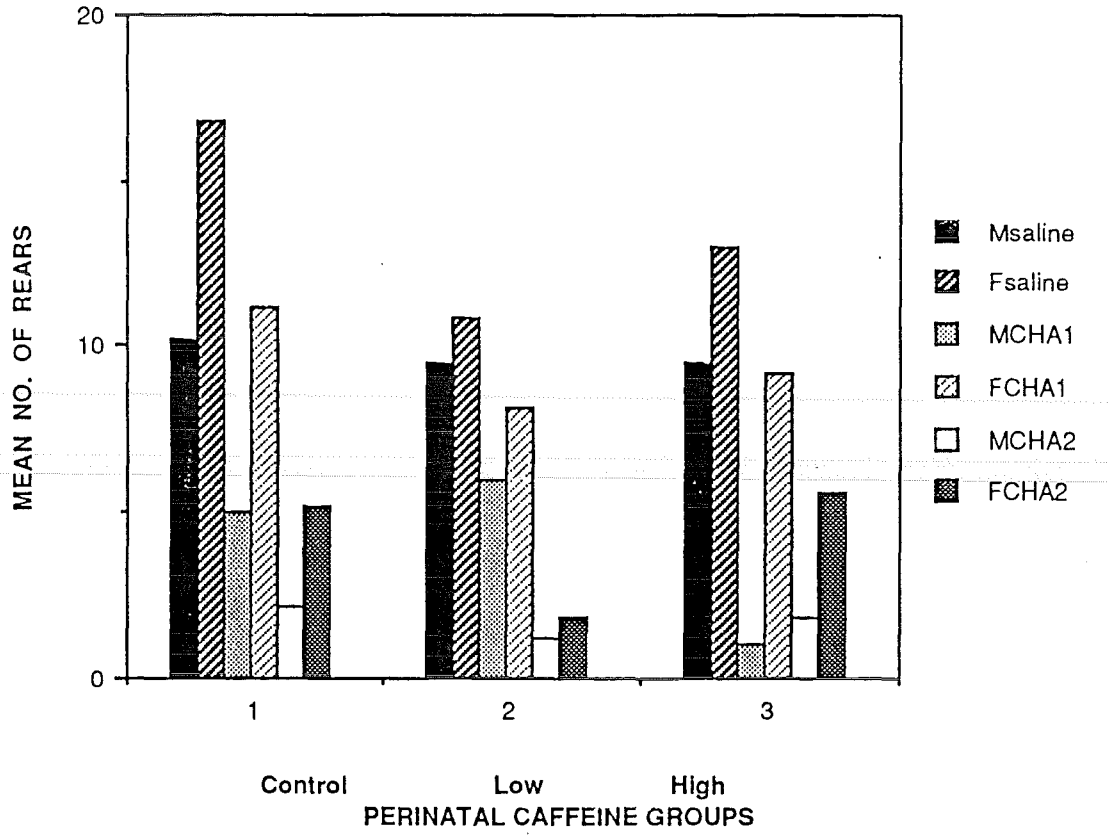



Figure 16(b). Effect of oxprenolol on rearing by rats perinatally exposed to caffeine

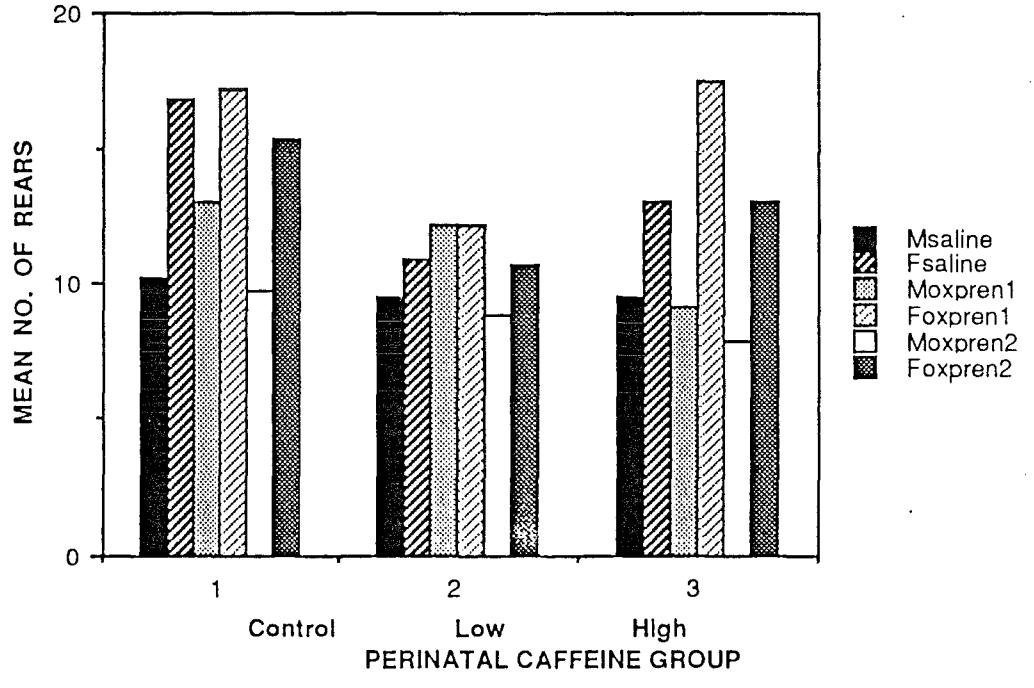


Figure 17(a). Effects of CHA on still behaviour by rats perinatally exposed to caffeine

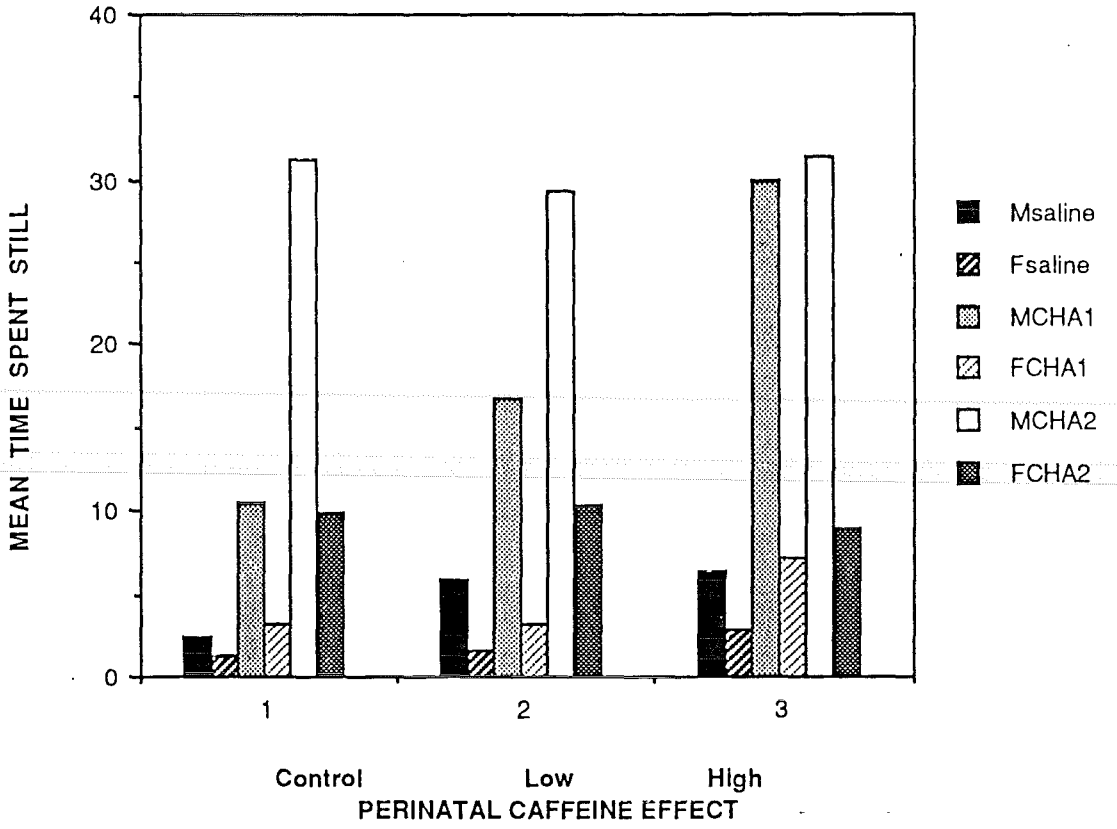



Figure 17(b). Effect of oxprenolol on still behaviour by rats perinatally exposed to caffeine

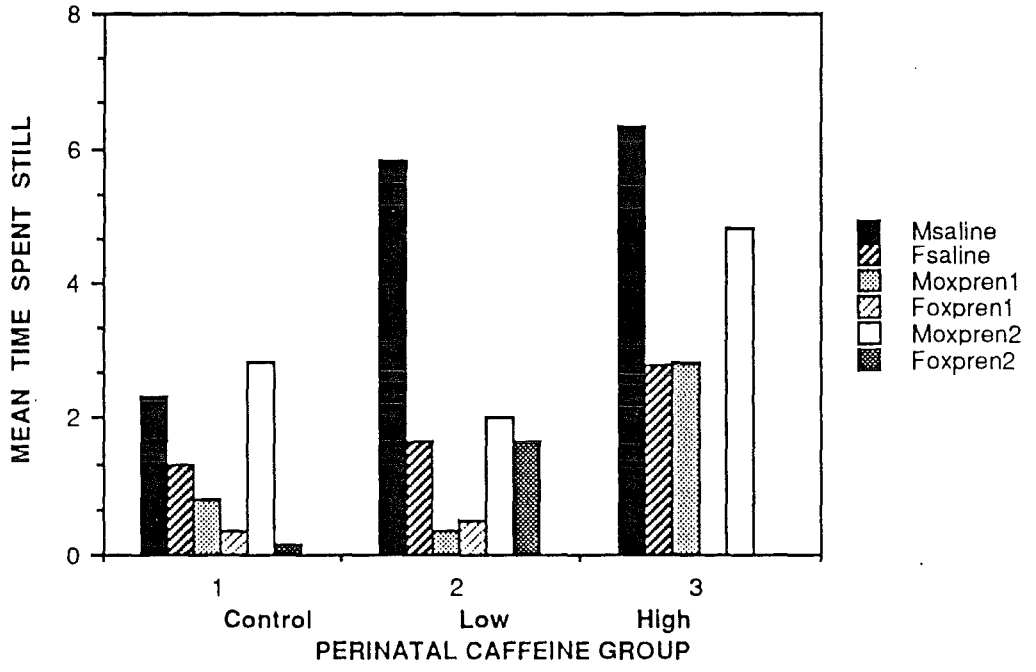


Figure 18(a). Effect of CHA on walking by rats exposed perinatally to caffeine

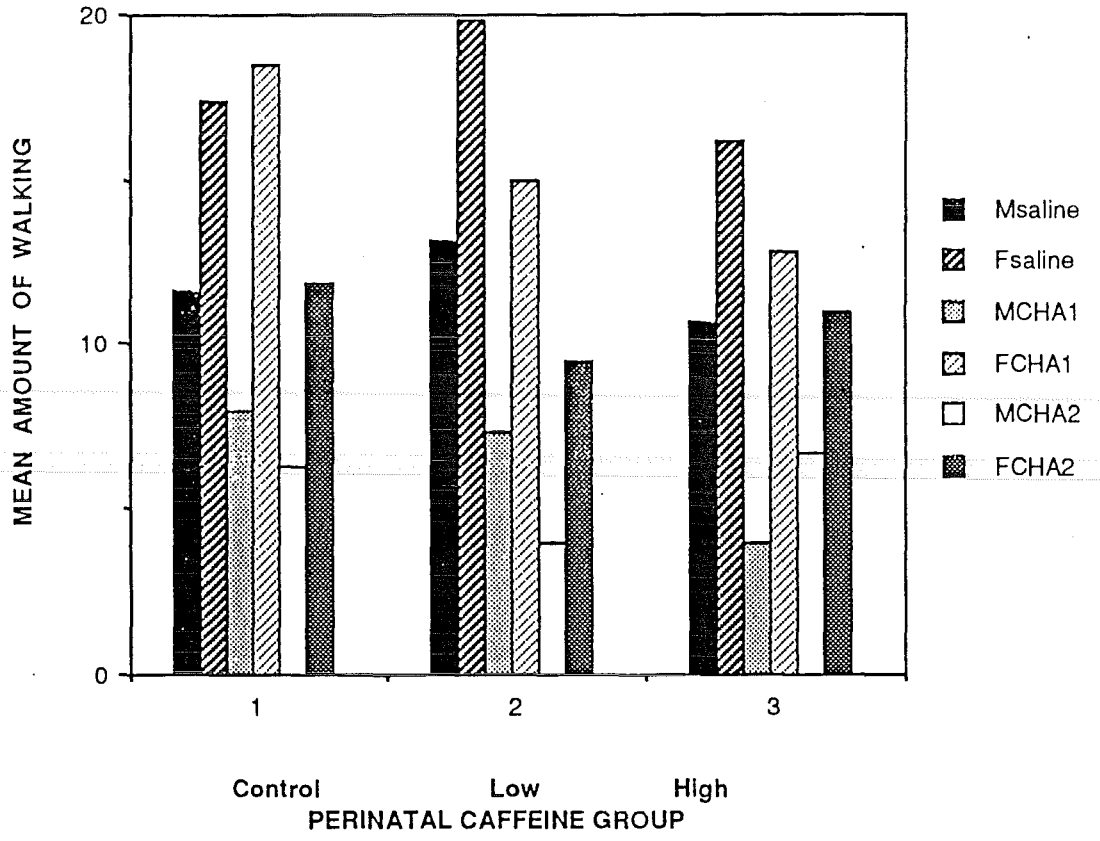
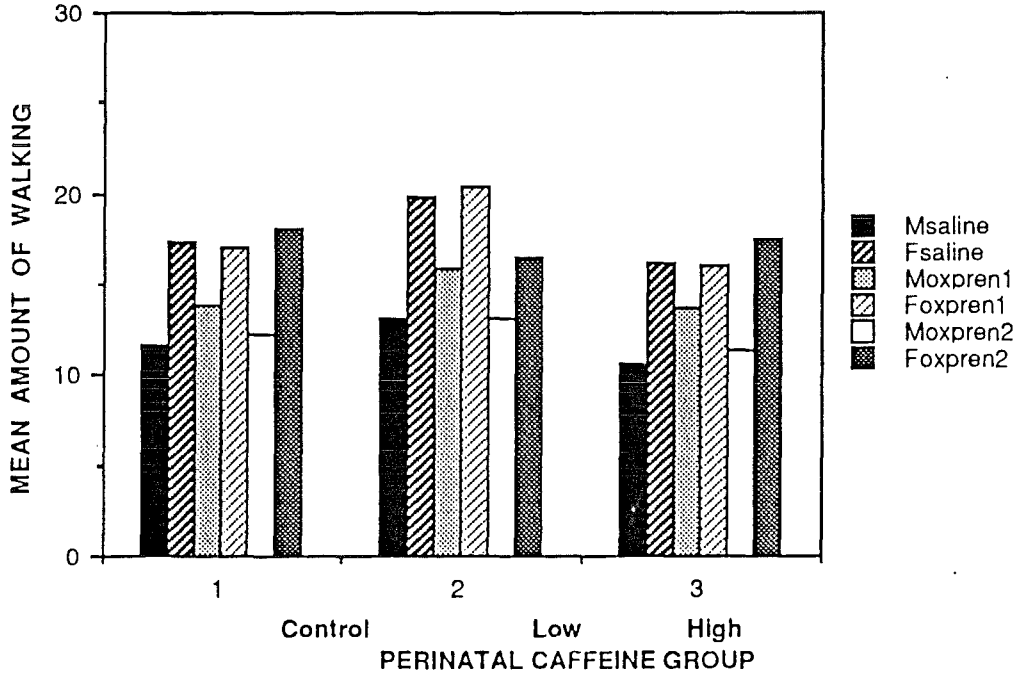



Figure 18(b). Effect of oxprenolol on walking by rats perinatally exposed to caffeine



# CHAPTER FOUR
# DISCUSSION

Many significant effects and trends emerged in this study. These will be considered in this discussion which comprises five parts. The first part examines the significant results to emerge in the first experiment and the differences between diazepam and caffeine on rat behaviour. The second part discusses CHA and oxprenolol's effects. Then some general findings overall will be examined. The third part will entail considering other factors which may be involved while the fourth part is a consideration of the brain mechanisms influencing behaviour. The last section will discuss general findings and some suggestions for future research will be made.

## EXPERIMENT ONE

Diazepam and the acute caffeine doses resulted in different behavioural responses. Caffeine gave a largely stimulant effect for the measures showing activity: ambulation, time spent still and walking. Diazepam however showed decreased activity for measures of ambulation, rearing, still and walking.

Dose effects occurred with diazepam for the high dose only, whereas with acute caffeine treatment, dose effects occurred for both the low and high doses. This indicates the sedatory effect of diazepam at the higher dose. Sedation effects are known to occur with diazepam as part of the anti-anxiety effect (Wu and Coffin, 1984). Regarding the other measures, defecation was lowered at the high dose of diazepam in males whereas no such effect was found for caffeine. An increased latency to emerge was found with the high dose of diazepam while again, rats were not affected by

doses of caffeine. Lowered grooming was found with the caffeine at high dose. Higher centre occupancy and lower corner occupancy occurred at low and high doses of caffeine while diazepam decreased centre occupancy at high dose and decreased corner occupancy at the low dose.

These results can best be described in terms of stimulation by caffeine and locomotor depression by diazepam. Examining the effects of caffeine first, the direct activity measures showed increased activity. However, the decreased grooming and decreased corner and centre occupancy may also be due to this stimulation by caffeine since moving around more means that the animals are not staying in one place such as the corners or the centre of the apparatus. They would not be on one spot to groom either.

Perinatal caffeine effects are of great interest, and they occurred for several of the measures when caffeine was administered. Grooming was higher in the high perinatal caffeine group and rearing and walking were lower in the low and high perinatal caffeine groups. There was also a trend although not significant, for male defecation to be higher in the high perinatal caffeine group while female defecation tended towards being higher in the low perinatal caffeine group (see figure 4(b)).

A possibility for this difference is that rats previously exposed to caffeine perinatally may have developed a longlasting tolerance to caffeine's effects. Tolerance is known to develop to caffeine with chronic exposure and Lombardelli et al. (1984) have provided evidence for longterm tolerance after caffeine exposure has finished. Activity by the control rats was increased compared with the experimental rats. However this may not be the case with this experiment since the prenatally exposed rats given saline also showed lowered activity compared to control rats given saline, while experimental group rats in the Lombardelli et al. study had slightly higher activity than controls with saline treatment.

The difference may instead be a sign of higher emotionality and depressed activity which is found at higher levels of caffeine intake. The biphasic effect of caffeine found at different doses did not show up in this experiment. The lack of biphasic effects from the acute caffeine was not unexpected considering that the doses used were fairly low and within the stimulant dose range. The lack of a difference between the low and high perinatal caffeine groups, which has been found in past research by Hughes and Beveridge (1990), may be due to one of several reasons. There may not be any difference between the two groups this long after exposure and the age of the animals or the test procedure may be resulting in different behaviour. These possibilities will be discussed again in the section about factors influencing the study.

Diazepam doses led to many depressant effects but only at the high doses. Low doses gave behaviour which was similar to saline. Corner occupancy increased with the high dose of diazepam which may be due to decreased activity. Rats seem to stay in the corners more when they are not moving around much. The increased latency to emerge was also consistent with lowered activity and sedation. Lowered defecation may have reflected lowered fear with greater sedation.

One perinatal caffeine effect occurred for centre occupancy, namely the low and high perinatal caffeine exposed groups spent more time in the centre. This could have been due to lowered fear.

## EXPERIMENT TWO

While dose effects with CHA were evident, those for oxprenolol were few. CHA had a largely sedatory effect which is consistent with the reported effects of adenosine analogs (Dunwiddie,1985). The lower dose showed a lesser degree of sedation than the high dose. Oxprenolol did not have any

visible effects on the rats. It certainly did not sedate, which is an advantage of beta blocker use over other anxiolytic agents (Noyes, 1982).

Ambulation was reduced by the low and high dose of CHA but was higher with the low dose of oxprenolol than the high dose . Rearing and walking were lowered by CHA while oxprenolol had no effect on these responses. Defecation was lowered with both doses of both drugs. However, the dose effects interacted with perinatal caffeine effects for centre occupancy and grooming with CHA.

The results for oxprenolol are few but those that did occur suggest more of a stimulating role than a sedating one. Beta blockers are known to have effects on the peripheral symptoms of anxiety whereas the effect on the central nervous system is a matter of debate (Noyes, 1982). This experiment did not allow a distinction to be made between these two mechanisms to discover which is responsible for oxprenolol's actions. However the lack of results may suggest that the CNS is not being affected. If oxprenolol is reducing the somatic symptoms of fear then the rats may become more active in the way found, due to a relaxation of fear symptoms.

Several perinatal caffeine effects were found in rats treated with oxprenolol. Centre occupancy was higher for females in the high perinatal caffeine group, males in the low perinatal caffeine group defecated less and rearing was decreased for both sexes in the low perinatal caffeine group. All these effects suggest a decrease in fear for the low and high perinatal caffeine groups after oxprenolol administration. Although perinatal caffeine effects were found, the usefulness of beta blockers in animal research has been questioned, with suggestions that they do not affect animals. However, other researchers have found effects with beta blockers (Hughes, 1981). Nevertheless, this could explain the lack of results with this drug or it may have been that dose levels were too low to show many effects.

CHA affected the rats in a sedative way. Lowered locomotor activity was typical in rats, but it is interesting that CHA was the only drug for which an interaction between dose and perinatal caffeine occurred. This happened for centre occupancy and for grooming.

Rats in the high perinatal caffeine group spent more time in the centre of the open field with the high dose than with the low dose of CHA. Grooming was lowered in the high perinatal caffeine group with the high dose of CHA. These two measures are possible indices of fear and the rats behaviour in both these instances indicates lowered fear. Caution must be taken with the grooming measure however since the numbers of grooming responses were small therefore making the results less behaviourally significant.

Both measures were affected in the high perinatal caffeine group, giving added support for a greater effect of CHA on perinatally caffeine treated rats than other drug treatments. It may be that CHA is interacting in the adenosine system in the brain in some way to affect the behaviour more than other drug treatments. The other two anti-anxiety agents also increased centre occupancy for the high perinatal caffeine groups which was an interesting finding. They may have decreased fear as well, but it may be that CHA had a greater effect evidenced by the dose interacting with the perinatal caffeine effect.

## BRAIN MECHANISMS

The implications of these results for the effect on the brain by perinatal caffeine are minimal. The effects of all the drugs on behaviour, particularly on emotionality were small. Evidence of increased emotionality in the perinatal caffeine exposed groups was hard to find. In

particular, defecation, usually described as the best measure for fear, was not greatly affected. Diazepam-, CHA- and oxprenolol-treated rats did show a decrease in defecation with low and high doses of CHA and oxprenolol and with the high dose of diazepam. Although sedation may account for this to some extent, the decrease with oxprenolol, for which there was a perinatal caffeine effect, suggests that an anti-anxiety effect may be responsible as well.

Emergence was another disappointing measure. This failed to show any results other than sex differences. The one dose effect, which occurred for diazepam, was most probably due to sedation. This lack of results may be due to the testing procedure which will be mentioned in the next section.

Grooming was increased with acute caffeine in the high group more than the other two treatments which may indicate increased fear. When rats in the high perinatal caffeine group were given CHA, lower grooming was found at the high dose, perhaps showing a greater effect of CHA on these rats than the other groups. The amount of grooming actually recorded in these experiments was very low. As it was a fairly rare behaviour, this data may not give a very good indication of the effects of the drugs.

Centre and corner occupancy gave very interesting results. These measures are not considered to be very reliable indices of emotionality (Archer, 1973) but with diazepam, CHA and oxprenolol more centre occupancy did occur in the high perinatal caffeine group than in either of the other two groups. This is hard to explain but it is tempting to relate it to reduced emotionality especially since the acute caffeine doses did not show this effect. Also, the interaction of dose with the perinatal caffeine effect with CHA might suggest a greater influence of this drug on caffeine's effects.

If CHA can influence the behavioural effects of perinatal caffeine exposure, this gives support for the proposal that adenosine is being altered in the rat brain. The way in which this occurs is not fully known. Many researchers have found increased numbers of adenosine receptors with

caffeine exposure (Boulenger et al., 1983). As diazepam did not have much effect on the rats, it is unlikely that the benzodiazepine was affecting the adenosine receptor.

Increased receptors may not mean that increased extracellular adenosine is responsible for the anxiogenic effects found with caffeine. If this were so, the adenosine agonists would not be proposed as anxiolytic in action. This is a difficulty for the adenosine theory. Adenosine should not be expected to increase anxiety when it is known that it has sedative, relaxant properties. Perhaps this discrepancy can be resolved with better knowledge of the different receptors or it may be that adenosine is influencing other neurotransmitters, which are causing the effects. It seems more and more likely that not one brain system or neurotransmitter is involved in caffeine's effects but many are interacting to produce the effects. It has also been suggested that adenosine may be responsible for the decrease in activity while the anxiety effects may be mediated elsewhere. But it is also likely that the depressed activity found with the increased emotionality is secondary, that is, it may actually be a result of increased anxiety.

## FACTORS INFLUENCING THE STUDY AND CRITICISMS

### SEX EFFECTS

Worthy of mention is the great number of sex effects which revealed themselves in the experiments. These were not unexpected, since many investigators have reported similar sex differences in behavioural studies (Hughes and Beveridge,1986, 1990). Males are bigger and heavier than females are therefore usually found to be much slower. Males also defecate

a lot more than females which was found for every instance of the defecation measure. The emergence measure also revealed a longer latency to emerge by male rats on every occasion, most likely due to the lower activity of the males.

Males have also been reported to be more affected by some drugs than females are. Hughes and Beveridge (1991) found that males were more susceptible to caffeine effects when exposed either prenatally or postnatally alone and the effects generalized over to females as well only when caffeine was given during both periods.

This study also found some support for this male-only effect. The sex and perinatal or dose interactions involved female effects only in three out of ten interactions while five out of ten involved male-only effects. Although this is not overwhelming evidence for male only-effects, the rats tested in Hughes and Beveridge (1990) study were the only group exposed perinatally, when both sexes were affected by caffeine.

This sex difference may be due to a sex hormone or some other sex dependent trait. Holloway and Thor (1984) have provided some evidence for testosterone being involved. When testosterone was high, an increase in social investigation occurred with caffeine administration. This effect was with acute caffeine treatment so perhaps different situations might characterise chronic treatment and testosterone effects.

Gray (1982) gives a cautionary message in regard to sex differences. It may be the case that in rats, males show greater fear responses than females, but in humans this effect shows the opposite pattern with females being the most prone to fear and anxiety. However it is difficult to recognise purely biological effects in humans, with cultural patterns so influential. Nevertheless, it is important to remember that the rat effects may not be appropriate for extrapolation to the human situation.

## PROBLEMS IN THE RESEARCH

A problem with comparing this research with the human situation is the issue of brain development in both species. Caffeine was exposed to rats perinatally to mimic the situation in humans with mothers ingesting caffeine during pregnancy and when breast-feeding. This seems the most likely pattern of behaviour in caffeine consumption by mothers. However, gestation and lactation are not equivalent in rats and humans. The developmental stages of the brain differ with the rats brain continuing to develop for relatively longer after birth. The equivalent of gestation and lactation in the rat for humans may be gestation alone. Therefore the data on perinatal effects in rats may be more suitable for showing the possible effects with caffeine exposure during gestation alone in humans.

Hughes and Beveridge (1991) covered the possible influence of environmental factors on offspring by altered behaviour in mothers ingesting caffeine, by fostering the young. Another similar problem however concerns effects on the prenatal environment, apart from the caffeine reaching the fetus. There is the possibility that the mothers' altered behaviour by caffeine ingestion may have influenced their unborn offspring (Archer and Blackman, 1971).

Boulenger et al. (1986) found that prenatal stress as well as caffeine, alters adenosine receptors. Pohorecky et al. (1989) found prenatal stress affected behaviour and altered sensitivity to caffeine in regard to corner activity and rearing and it decreased gnawing activity. Therefore it is possible that the dams' behaviour may be affected by caffeine. West et al. (1986) found dams behaviour after caffeine exposure was changed in the form of increased activity, decreased weight gain and a lowered food intake.

If greater stress is experienced by the dams, the chemical and

neuroendocrine changes may be reaching and affecting the offspring. The knowledge that caffeine by itself does cause anxiety however makes it unlikely that prenatal stress alone is causing the changes in offspring behaviour, but they may have an additive effect. Controlling for this prenatal stress would be virtually impossible to do.

The amount of caffeine given to the rats has been a matter of debate as well. The doses given are extraordinarily high compared to the amount of caffeine consumed by people, Hughes and Beveridge (1990) estimated that a 28 mg/kg/day dose of caffeine is equivalent to about 16 cups of coffee in a 56 kg woman. This is very high, but if metabolic differences and surface area are taken into account the equivalent amount in the same 56 kg woman is about five and a half cups of coffee, which is far closer to the real situation.

The design of the experiments may have been responsible for some lack of effects. The main problem in the design was the use of a repeated measure procedure. Although an attempt was made to guard against the repeated testing of rats influencing their behaviour (by testing each rat a week after its previous test), it seems as if this may have happened. Archer (1973) reported many examples of repeated testing causing an adaptation effect. These were usually studies that tested the rats every day, the testing arena became a familiar place which changed the behaviour.

The number of rats used in this study made it difficult to assess the possibility of adaptation by the rats, especially with the method employed of testing all rats in one cage in succession. However the emergence data did seem to confirm that the repeated measure was obscuring results. A each rat had more exposures to the emergence apparatus, it became more likely that they would emerge. Very few emerged on the first trial but most had by their fifth trial. In the second experiment rats were not tested as often as in

the first, more rats failed to emerge in the second experiment overall, reflecting the longer periods away from (and possibly lower familiarity with) the apparatus (compare figure 14 with figure 5). If familiarity has occurred then it seems inevitable that it would influence behaviour. In spite of such influences, it is clear that some effects still occurred suggesting that they were fairly robust.

Fear from the testing procedure itself is likely to have occurred. It has been shown that bright light and noise are fearful stimuli in the open field for rats (Walsh and Cummins, 1976, Archer, 1973) although a relationship between bright light and defecation does not seem to exist (which seems unusual since defecation is regarded as the major fear index). White noise has been shown to decrease locomotion but it has also been reported to increase locomotion and defecation at levels over 90 dB.

White noise of 58 dB and the fluorescent tube light were employed in this study to control extraneous variables. The light kept a constant amount of illumination over the entire open field area and the white noise was needed to mask background noise. All rats were exposed to an equal amount of white noise and light.

An attempt was also made to exclude fear of the experimenter. Rats were handled daily for several weeks before the commencement of the experiment. However, the injection of the rats may have caused some fear reactions, particularly since five injections in total were given to each rat and an association of the injection with the experimenter may have developed. Testing each rat in succession from each cage may have caused more fear in the rats, injected rats may have influenced those to be injected or all injected rats may have developed the same emotional state.

Age of the rats is also a matter of interest. These rats were tested at 8 to 11 months after birth. Decreases in activity and greater emotionality

have often been found as age increases (Hughes and Beveridge, 1986). However, Hughes and Beveridge (1990) found age-related increases in ambulation and rearing but decreased walking. It is difficult to see effects of age in these experiments as sedation was so marked. Perhaps sedation was greater due to the age of the animals.

The measures used in this study are the final problem that will be mentioned. What is actually being measured is a matter of concern. Emotionality is very difficult to assess in animals particularly when defined as "anxiety". Fear in rats may be possible to measure but is still subjective. The behavioural measures were more likely to measure fear which was possibly similar to anxiety in humans. Therefore the terms are often used interchangeably.

The emotionality or fear measures used in this study were defecation (the most valid index), corner or centre occupancy, emergence from a dark box to an illuminated area and grooming, which is probably the weakest measure involving a type of displacement behaviour when fearful. However it may just be a behaviour which is performed when the rat is not doing anything else.

The activity measures while also relating to the fear measures, are often described as exploratory or escape behaviours. Ambulation, walking and rearing have all been treated as exploratory measures when they may not be (Archer, 1973). Escape behaviour might be best measured by fast ambulation when a rat is first placed in the open field, or rearing against the sides of the open field may be an escape behaviour. It is difficult to distinguish between escape and exploratory behaviours but with this distinction existing it may be that different behavioural measures are controlled in different brain areas.

The relatedness of the measures are of interest with this point in mind. All three measures (as well as time spent still showing the inverse

relationship), show a similar pattern of effects. There was a decrease in activity with diazepam and CHA administration. Ambulation was increased with oxprenolol at the low dose, less time was spent still with the low dose while no dose effect occurred for rearing or walking. Higher rearing was found in the control group, the low group had decreased rearing.

Caffeine also gave mixed results. Higher walking and higher ambulation occurred at low and high dose, less time was spent still but again no dose effect was found for the rearing measure. But lower rearing and lower walking occurred for the two perinatal caffeine groups. Rearing may be separate from the other measures but it did relate to walking in the perinatal caffeine effects. Therefore this experiment was not rigorous enough to distinguish any differences in the activity measures, although Hughes and Beveridge (1991) have suggested that a difference between walking and ambulation that they found may reflect different underlying mechanisms. In this type of study it may be better to merely consider these measures as activity measures and not consider exploration and escape functions.

## GENERAL CONCLUSIONS AND FUTURE RESEARCH

The most interesting results occurred for the measure of centre occupancy, particularly with CHA administration. Dose interacted with perinatal caffeine group showing a significantly greater amount of time spent in the centre of the open field than in the periphery of the arena, for the high perinatally exposed group given the high dose of CHA. Higher centre occupancy also occurred in the high perinatal caffeine groups with diazepam and oxprenolol administration but this was not dose specific.

This leads to a tentative suggestion that they are all reducing anxiety in the high perinatal caffeine groups but that in the CHA treatment the effect is more pronounced, suggesting a greater influence of this drug on rats behaviour. This could be due to the adenosine system in the brain being more affected in rats exposed to the high dose of caffeine throughout gestation and lactation.

Reduced activity was evident with diazepam at the high dose only and with CHA at both the high and low dose, the low dose being intermediary between saline and the high dose. This was due to the sedative action of both drugs. Oxprenolol stimulated activity which may be due to somatic fear symptoms being relieved. Caffeine had a largely stimulating effect except for the finding of reduced activity by males in the low and high perinatal caffeine groups for the measure of walking. Higher grooming and rearing was found in the high perinatal group which is suggestive of increased emotionality/fear.

This study was fairly limited in that the brain mechanisms involved were not immediately accessible. A further study of a wide range of behavioural effects from perinatal caffeine exposure needs to be made with an examination of brain slices to find changes in the brain adenosine receptors and of other possible receptors involved. Levels of neurotransmitters such as NE and seratonin should be measured to find out if they are being altered. This may be related to receptor numbers in the brain. It may be that increases in adenosine are influencing, most probably inhibiting, neurotransmitter systems so these levels may correlate. There is a lack of studies measuring behavioural changes in the brain after perinatal caffeine exposure relating this to the brain abnormalities or differences which have been found. Both aspects need to be examined in the same rats.

The implications of upregulated adenosine receptors are not clear.

Upregulation does not seem to increase the amount of adenosine in the brain or the effect would be a decrease in anxiety. This obviously needs to be further examined. When more is understood about adenosine, the problem of perinatal caffeine and behavioural effects may be better understood.

# ACKNOWLEDGEMENTS

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